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Conversion of human 15-lipoxygenase to an efficient 12-lipoxygenase: the side-chain geometry of amino acids 417 and 418 determine positional specificity

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Positional specificity determinants of human 15-lipoxygenase were examined by site-directed mutagenesis and by kinetic analysis of the wild-type and variant enzymes. By comparing conserved differences among sequences of 12- and 15-lipoxygenases, a small region responsible for functional differences between 12- and 15-lipoxygenases has been identified. Furthermore, the replacement of only two amino acids in 15-lipoxygenase (at 417 and 418 in the primary sequence) by those found in certain 12-lipoxygenases results in an enzyme that has activity similar to 12-lipoxygenase. An examination of the activity of nine variants of lipoxygenase demonstrated that the amino acid side-chain bulk and geometry of residues 417 and 418 are the key components of the positional specificity determinant of 15-lipoxygenase. Overexpression of a variant (containing valines at positions 417 and 418) that performs predominantly 12-lipoxygenation was achieved in a baculovirus–insect cell culture system. This variant was purified to >90% homogeneity and its kinetics were compared with the wild-type 15-lipoxygenase. The variant enzyme has no change in its apparent K_M for arachidonic acid and a minor (3-fold) change in its V_{max}. For linoleic acid, the variant has no change in its K_M and a 10-fold reduction in its V_{max}, as expected for an enzyme performing predominantly 12-lipoxygenation. The results are consistent with a model in which two amino acids of 15-lipoxygenase (isoleucine 417 and methionine 418) constitute a structural element which contributes to the regiospecificity of the enzyme. Replacement of these amino acids with those found in certain 12-lipoxygenases results in an enzyme which can bind arachidonic acid in a catalytic register that prefers 12-lipoxygenation.

Key words: arachidonic acid/conserved differences/lipoxygenase/regiospecificity/substrate specificity

Introduction

The lipoxygenases are a family of dioxygenases which act on fatty acids containing a 1,4-*cis*,*cis*–pentadiene. The product of this reaction is a fatty acid hydroperoxide, containing a 1-hydroperoxy-2,4-*trans*,*cis*–pentadiene. Lipoxygenases may be important targets for drug design because they have been implicated in the pathogenesis of a variety of inflammatory conditions such as arthritis, psoriasis and bronchial asthma (Samuelsson *et al.*, 1987). Furthermore, 15-lipoxygenase has been implicated in the early stages of atherosclerosis, as it is induced in human atherosclerotic lesions (Yla-Herttuala *et al.*, 1990) and is capable of oxidizing low-density lipoprotein to its atherogenic form (Parthasarathy *et al.*, 1989; Steinberg *et al.*, 1989).

The primary sequences of the major mammalian lipoxygenases have a high degree of identity, yet these enzymes differ in their positional specificity on arachidonic acid. Thus, 5-lipoxygenase catalyzes the dioxygenation of the carbon-5 of arachidonic acid, 12-lipoxygenase acts on carbon-12, and 15-lipoxygenase acts on carbon-15. An understanding of the structural features of these enzymes, which are responsible for the difference in positional specificity, will provide information about important enzyme–substrate interactions. Such information can come from a combination of site-directed mutagenesis studies, direct chemical modification of the active site of the enzyme and X-ray crystallography. The only known 3-D structure of any lipoxygenase is that of soybean isoform-1 (Boyington *et al.*, 1993). There are two solvent-accessible cavities in the structure: one proposed to be an oxygen tunnel, the other the fatty acid cavity. However, this structure was solved in the absence of any ligands, and therefore it is difficult to assess the importance of particular regions of the structure for the enzyme's activity.

Although the catalytic mechanism of the enzyme has yet to be described fully, it has been shown that the first rate-limiting step of the reaction is the stereospecific abstraction of hydrogen from a bis-allylic methylene (Hamberg and Samuelsson, 1967). Molecular oxygen is then inserted stereospecifically two carbons away from the site of the hydrogen abstraction, resulting in a hydroperoxy-fatty acid containing a conjugated diene. The activity of rabbit 15-lipoxygenase has been analyzed for fatty acids of various lengths and of various degrees of saturation. It was found that the rabbit enzyme prefers substrates which have a bis-allylic methylene eight or nine carbons away from the methyl terminus of the fatty acid substrate, implying that this distance is the key determinant in the positional specificity of the enzyme (Kuhn *et al.*, 1990b). Furthermore, it has been demonstrated that both the rabbit (Murray and Brash, 1988; Kuhn, *et al.*, 1990a) and the human 15-lipoxygenase (Kuhn, *et al.*, 1993) have activity on various membrane phospholipids. Thus it is likely that a key enzyme–substrate interaction is present at the methyl terminus of the fatty acid, so that the ω-8 carbon (the eighth carbon from the methyl end) is positioned near the reaction center for hydrogen abstraction. If this carbon is a bis-allylic methylene (as in arachidonic acid and linoleic acid), hydrogen can be abstracted and the reaction can proceed. Recently, it has been demonstrated that the 12-lipoxygenase from porcine leukocytes can oxidize membrane phospholipids, suggesting that a key enzyme–substrate interaction for this enzyme is also present at the methyl terminus of the substrate (Takahashi *et al.*, 1993).

Previously, we searched the sequences of 12- and 15-lipoxygenases to determine which amino acids may be responsible for the positional specificity difference of these two enzymes. We found four conserved differences in the sequences, i.e. four amino acids were conserved in the three 12-lipoxygenase sequences at sites that not only differed from

the 15-lipoxygenase sequences but were also conserved within the 15-lipoxygenase sequences. We have demonstrated by site-directed mutagenesis that one of these conserved differences in human 15-lipoxygenase is important in determining the positional specificity of the enzyme. Replacement of this amino acid in 15-lipoxygenase (methionine at 418) with the amino acid found in the 12-lipoxygenases (valine) results in an enzyme that catalyzes equally 12- and 15-lipoxygenation. Furthermore, replacement of this methionine as well as the two neighboring amino acids with amino acids found in the 12-lipoxygenase of human platelets results in an enzyme with activity closely resembling that of 12-lipoxygenase (Sloane *et al.*, 1991). However, due to limitations in the levels of expression in *Escherichia coli*, previous studies were performed using bacterial crude lysates. Hence, the kinetic constants of a purified mutant have not been measured. Furthermore, the structural requirements of positional specificity have remained undefined.

By primary sequence alignment, methionine 418 in 15-lipoxygenase is analogous to an amino acid (phenylalanine 557) in a solvent-accessible cavity of the soybean enzyme (Boyington *et al.*, 1993). The results of our mutagenesis study imply that this cavity is the fatty acid substrate binding cavity; however, an interaction between this amino acid and the fatty acid substrate cannot be deduced rigorously because the structure was solved in the absence of a substrate. Our results led to a model for positional specificity in which methionine 418 defines the binding pocket of the methyl terminus of the fatty acid substrate. Consistent with this model is the possibility that the amino acid side chain at position 418 interacts directly with the substrate; thus, the hydrophobicity and bulk of the side chain would be crucial elements in determining the positional specificity of the enzyme. The replacement of this amino acid with smaller hydrophobic amino acids would define a deeper pocket, allowing the fatty acid substrate to bind deeper into the enzyme, in a catalytic register that is optimal for 12-lipoxygenation. We have tested the predictions of this model by introducing various amino acid replacements at this site. Furthermore, we overexpressed and purified one critical mutant to analyze the enzyme's kinetics in more detail. The results we present here are consistent with a model in which the amino acids at positions 417 and 418 define the positional specificity of the enzyme through conformational or configurational parameters, rather than through direct side chain–substrate interactions. Furthermore, the kinetics of the enzyme have not been altered, implying that these mutations have not affected the catalytic efficiency of the enzyme; rather, they allow the substrate to bind in the enzyme in a register that favors 12-lipoxygenation.

Materials and methods

Materials

Bacterial strains. All *E.coli* expression was performed in strain DH5α [F^- *endA1*, *hsdR17* ($r_k^-m_k^+$), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*, Δ(*argF–lacZYA*), *U169*, ϕ80 *dlac* Δ*lacZ* ΔM15]. Uracil-laden single-stranded DNA was prepared from strain LE112 (F′ *lacI*^{q}, *lacZ*::*Tn5*, *proAB–dut1*, *ung1*, *relA1*; Evnin *et al.*, 1990). Helper phage for the single-stranded DNA preparation was VCSM13 from Stratagene (La Jolla, CA).

Enzymes and molecular biology reagents. All restriction endonucleases and deoxynucleoside triphosphates were purchased from Boehringer-Mannheim (Indianapolis, IN). T4 DNA polymerase and T4 DNA ligase were obtained from Promega (Madison, WI). DNA polymerase I (Klenow fragment) was acquired from Bethesda Research Laboratories (Gaithersburg, MD). All reagents were of the highest commercial grade.

Plasmids and oligonucleotides. The plasmid pSS15LO (De Marzo *et al.*, 1992) was used for all mutagenesis and bacterial expression. For baculovirus–insect cell culture expression we used the transplacement vector pSynXIV VI$^+$ X3 (Wang *et al.*, 1991). All oligodeoxynucleotides were synthesized on an Applied Biosystems 308B DNA synthesizer at the Biomolecular Resource Center, University of California, San Francisco, CA.

Other reagents. Arachidonic acid (eicosa-5Z,8Z,11Z,14Z-tetraenoic acid), linoleic acid (octadeca-9Z,12Z-dienoic acid), eicosa-11Z,14Z,17Z-trienoic acid, eicosa-5Z,8Z,11Z-trienoic acid, eicosa-8Z,11Z,14Z-trienoic acid, eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid, docosa-4Z,7Z,10Z,13Z,16Z,19Z-hexaenoic acid, and 12- and 15-hydroxyeicosatetraenoic acid (HETE) were purchased from Biomol (Plymouth Meeting, PA). 13-Hydroperoxyoctadecadienoic acid was purchased from Oxford Biomedical Research (Oxford, MI).

Site-directed mutagenesis

Plasmid construction and bacterial expression. For all site-directed mutagenesis and bacterial expression studies, the plasmid pSS15LO was used (De Marzo *et al.*, 1992). This plasmid contains an origin of single-stranded replication from pUC-f1 (Pharmacia) to facilitate site-directed mutagenesis. Transcription of the cDNA encoding 15-lipoxygenase is promoted by the *lacZ* promoter of pSS15LO. The start codon (AUG) of the 15-lipoxygenase is placed an optimal distance from the *lacZ* ribosome binding site to achieve bacterial expression.

Mutagenesis. Site-directed mutagenesis was performed by the method of Kunkel (1985), as modified previously (Evnin and Craik, 1988). Uracil-laden single-stranded pSS15LO was isolated from *E.coli* LE112 which had been freshly transformed with double-stranded pSS15LO and infected with helper phage VCSM13 by standard methods (Sambrook *et al.*, 1989). DNA was purified from phage proteins as follows. The phage proteins were suspended in a 2% SDS solution and heated to 70°C for 10 min. The SDS–protein was then precipitated by the addition of an equal volume of 3 M potassium acetate and 5 M acetic acid, and centrifugation at 12 000 *g* for 15 min. The resulting supernatant was then extracted once with an equal volume of phenol saturated with TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), twice with an equal volume of phenol:chloroform (1:1), and once with an equal volume of chloroform. The DNA was precipitated from the final aqueous phase by the addition of an equal volume of isopropyl alcohol. The precipitated DNA was then resuspended in TE. The template for the mutagenesis reaction prepared in this way would typically yield a 60–70% mutation frequency.

Synthetic oligodeoxynucleotides were annealed to the single-stranded template at a molar ratio of 25:1 (primer:template). T4 DNA polymerase was used for the *in vitro* DNA synthesis, as described (Evnin and Craik, 1988). To make the I417V change, we used an oligodeoxynucleotide with the sequence 5′-CCAGTACTCATAACCTGGTCG-3′. For the double mutant I417V,M418V, we used an oligodeoxynucleotide with the sequence 5′-CCAGTACTAACTACCTGGTCG-3′. To replace the methionine at position 418 with asparagine, lysine, iso-

leucine, leucine, threonine or tryptophan, we used the redundant oligodeoxynucleotide 5′-CCAGTACT(ACT)(GATC)(TA)TATCTGGTCG-3′. The above oligodeoxynucleotides were complementary to nucleotides 1245–1265 in the human 15-lipoxygenase cDNA sequence (Sigal *et al.*, 1988). The bold bases indicate mismatches and bases in parentheses are redundant at that position. For generating the quadruple mutant 15LOX (Q416K,I417A,M418N,S419A), an oligodeoxynucleotide with the sequence 5′-CCTCCCCCACCAGTAGCGTTAGCTTTGTCGAAAATTCCC-3′ was used. This oligodeoxynucleotide is complementary to nucleotides 1236–1274 in the published sequence. Each oligodeoxynucleotide is designed to not only make the desired change in the amino acid sequence, but also change the recognition sequence of the restriction endonuclease Hgi AI (5′-GAGCAC-3′, at position 1257 of the published cDNA sequence), rendering the mutated DNA resistant to cleavage by Hgi AI at this site, to facilitate screening. To make the 12LOX (V419M) and 12LOX (V418I,V419M) mutations, we used an oligodeoxynucleotide with the sequence 5′-CCTGTGCTCATTA(TC)CTGGTCG-3′, which is complementary to nucleotides 1245–1265 in the published sequence of the bovine 12-lipoxygenase cDNA (De Marzo *et al.*, 1992). This oligodeoxynucleotide not only makes the desired changes in the amino acid sequence of 12-lipoxygenase, but also mutates an existing *Hph*I restriction endonuclease recognition sequence for screening. The plasmid pSS12LO (De Marzo *et al.*, 1992) was used for the mutagenesis and expression of 12-lipoxygenase. All mutations were first identified by an alteration in the restriction map of the plasmid, then confirmed by DNA sequencing using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), modified for double-stranded DNA (Sequenase kit; USB, Cleveland, OH).

Baculovirus expression. The expression of wild-type human 15-lipoxygenase in a baculovirus–insect cell culture system has been described previously (Kuhn *et al.*, 1993). To express the 15LOX (I417V,M418V) variant in baculovirus, the transplacement vector pSyn15vv was constructed. A 2.7 kb fragment of pSS15LO, containing the 15LOX (I417V,M418V) coding region, was isolated by digestion with restriction endonucleases *Nde*I and *Eco*RI. This fragment was purified by agarose gel electrophoresis and glass-powder elution (Geneclean kit, Bio 101, La Jolla, CA), then ligated to the baculovirus transplacement vector pSynXIV VI$^+$ X3 (Wang *et al.*, 1991) which had been linearized by digestion with *Eco*RI and treated with calf intestinal phosphatase. The ligation reactions were started by the addition of T4 DNA ligase and incubation at 15°C for 16 h. The 3′ ends of the resulting DNA were made flush by adding the large fragment of *E.coli* DNA polymerase I and 10 mM deoxynucleoside triphosphates, and incubating at 15°C for 1 h. After extraction with phenol and chloroform, T4 DNA ligase was added and the solutions were incubated at 15°C for 16 h. This mixture was then used to transform competent *E.coli* DH5α (Hanahan, 1983). Plasmid DNA prepared from the transformants was then screened for the correct orientation of the 15LOX (I417V,M418V) coding region by digestion with *Bgl*II. One of the plasmids identified in this way was used to re-transform *E.coli* DH5α for large-scale plasmid preparation (Qiagen, Chatsworth, CA) for DNA sequencing and transfection into Sf-9 cells, as described previously (Kuhn *et al.*, 1993).

Protein purification. 15LOX (I417V,M418V) was purified from the baculovirus–insect cell culture by anion exchange chromatography, as described previously for the wild-type 15LOX (Kuhn *et al.*, 1993) except that the chromatography system used was a Waters 650 Advanced Protein Purification System. Column chromatography was performed on a Waters Protein Pak Q8 HR semi-preparative AP-1 (10×100 mm) column (Millipore, Milford, MA).

Lipoxygenase assay, enzyme kinetics and HPLC analysis. To analyze the activity of variant lipoxygenases in the bacterial expression system, 3 ml cultures were grown at 37°C overnight in LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 80 μg/ml ampicillin. The bacterial cells were harvested by centrifugation at 5000 *g* for 10 min and resuspended in 1 ml PBS (1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, 0.15 M NaCl). Arachidonic acid (100 μg) was added and the resuspended cells were incubated at 37°C for 15 min. The cells were then placed on ice and sonicated using a Branson sonifier at a low setting three times (30 s each). The sonicated cell mixtures were then extracted for HPLC analysis as described previously (De Marzo *et al.*, 1992). The reverse-phase HPLC analysis was performed as described (Sigal *et al.*, 1990). The activities of wild-type 15-lipoxygenase and wild-type 12-lipoxygenase, expressed in *E.coli*, were analyzed by this technique for comparison. The ratio of products (12-HETE:15-HETE) produced by the wild-type 15-lipoxygenase was typically 1:9, and by the wild-type 12-lipoxygenase was typically 15:1.

The determination of the enzyme kinetics was performed on protein purified from the baculovirus–insect cell culture system as described (Kuhn *et al.*, 1993), with the following changes. The reactions were performed in a quartz cuvette at 4°C and the absorbance at 234 nm was monitored continuously for 3 min on a Beckman DU650 spectrophotometer. The linear part of the reaction progress curve was used to calculate the reaction rates using a molar extinction coefficient for conjugated dienes at 234 nm of 23 000 M/cm (Gibian and Vandenberg, 1987). Values for apparent K_M and V_{max} were estimated from Lineweaver–Burk and Hanes–Woolf analyses, using a best-fit linear regression by the CricketGraph III program on a Macintosh computer.

Results

Mutagenesis

By searching for conserved differences in the sequences of the 12- and 15-lipoxygenases, we have been able to demonstrate that methionine 418 in human 15-lipoxygenase is a key determinant for positional specificity. Further mutations demonstrated that replacing amino acids at positions 416, 417 and 418 of 15-lipoxygenase with those found in the 12-lipoxygenase of human platelets resulted in an enzyme with the positional specificity of 12-lipoxygenase (Sloane *et al.*, 1991). To determine which of these three amino acids are required as determinants for the positional specificity of the enzyme, we changed the amino acids at positions 417 and 418 from those found in 15-lipoxygenase (isoleucine and methionine respectively) to those found in a 12-lipoxygenase (valines at both positions). The bacteria expressing this double mutant lipoxygenase [15LOX (I417V,M418V)] were incubated with arachidonic acid, sonicated and the fatty acids extracted for reverse-phase HPLC analysis. The predominant peaks of the chromatogram, monitored at 234 nm, were coeluted with authentic 12- and 15-HETE (data not shown) in a ratio of 20:1 (12-HETE:15-HETE), a ratio similar to wild-type 12-

Table I. The effect of different amino acids at positions 417 and 418 of human 15-lipoxygenase on the product profile of the enzyme

Amino acid 417	Amino acid 418	12-HETE:15-HETE
Ile	Met (wild-type)	1:9
Ile	Val	1:1
Val	Met	1:1
Val	Val	20:1
Ile	Asn	1:1
Ile	Lys	1:9
Ile	Ile	1:1
Ile	Leu	1:9
Ile	Thr	1:3
Ile	Trp	not active

lipoxygenase. When either isoleucine 417 or methionine 418 was changed to valine alone, the resulting activity yielded equal 12- and 15-HETE (Table I).

A series of mutations was carried out at position 418 to determine how the different chemical characteristics of certain amino acids at this position would affect the enzyme's activity (Table I). Replacing the methionine with asparagine resulted in an enzyme with equal 12- and 15-lipoxygenase activity. The activity of the methionine to lysine mutant was indistinguishable from wild-type 15-lipoxygenase. To examine the effect of the geometry of the amino acid side chain, we replaced the straight-chain methionine at 418 with a branched-chain isoleucine. The resulting activity yielded equal 12- and 15-HETE. In contrast, mutating the methionine into a leucine, which is branched at the γ carbon, had no effect on the 15-lipoxygenase activity. Further mutagenesis introduced a threonine at position 418, resulting in an enzyme which predominantly performs 15-lipoxygenation but with a product ratio (1:3 12-HETE:15-HETE) that indicates increased 12-lipoxygenation (Table I). Finally, replacing the methionine at 418 with a tryptophan resulted in the expression of inactive protein.

A quadruple mutation was carried out, converting the QIMS sequence at positions 416–419 to KANA to introduce the 5-lipoxygenase sequence at this region. The *E.coli* expressing this variant lipoxygenase had no detectable activity on either arachidonic acid or linoleic acid (data not shown). All mutant cultures were assessed for protein expression by SDS–PAGE, followed by immunoblot detection. To the limits of this technique, the levels of expression were not altered in any of the mutants (data not shown).

Purification and enzyme kinetics

The levels of expression of the wild-type and mutated lipoxygenases in *E.coli* are sufficient to analyze the products formed from the incubation with arachidonic acid; however, multiple attempts to purify the enzymes from *E.coli* were unsuccessful, presumably due to the low levels of expression. To increase the levels of expression, we subcloned the 15LOX cDNA into various *E.coli* expression vectors which contained a tandem *tac* promoter, a promoter from the bacteriophage T7 or the *phoA* promoter. Also, a bicistronic expression system in which the lipoxygenase expression is linked to the highly expressed cheY protein was constructed. None of these other vectors resulted in increased expression. Previously, the wild-type 15-lipoxygenase had been successfully overexpressed and purified from a baculovirus–insect cell culture system (Kuhn *et al.*, 1993). We constructed a baculovirus transplacement vector (pSyn15vv) which encodes the 15LOX (I417V,M418V) variant

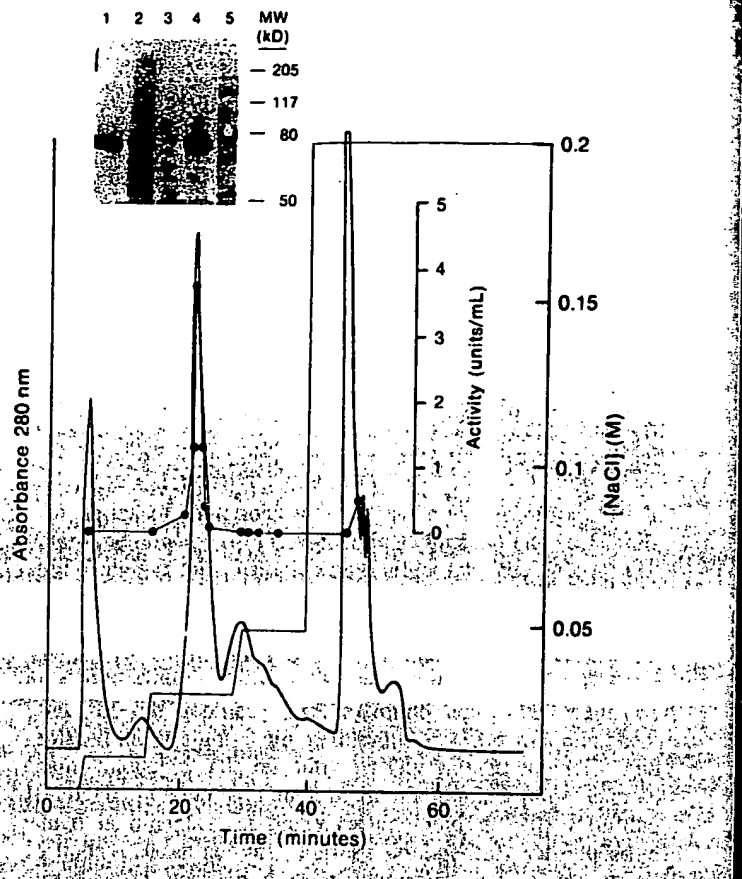

Fig. 1. The purification of 15LOX (I417V,M418V) by anion exchange chromatography. Insect cell lysates were prepared as described (Kuhn *et al.*, 1993). 2 ml of the insect cell lysate (20 mg/ml) were injected onto a Protein Pak Q8 HR semi-preparative column (Millipore, Milford, MA). The column was developed at a flow rate of 2 ml/min, using increasing percentages of buffer B (50 mM Bis–Tris, pH 6.8, containing 0.2 M NaCl) as indicated by the gradient line. The column effluent was monitored continuously at 280 nm. Fractions (2 ml) were collected and lipoxygenase activity was measured using the spectrophotometric assay, with arachidonic acid as a substrate (●). Inset: SDS–PAGE (7.5%) stained with Coomassie brilliant blue. Protein lanes are: lane 1, 5 μg purified wild-type human 15-lipoxygenase (Kuhn *et al.*, 1993); lane 2, 10 μg insect cell lysate infected with baculovirus containing 15LOX (I417V,M418V) cDNA; lane 3, 10 μg column flow-through (fractions 4–10); lane 4, 10 μg fraction 23; and lane 5, 10 μg fraction 48.

to achieve high levels of expression for purification and enzymatic studies. Cultures infected with baculovirus, recombined with pSyn15vv, contained a large amount of protein (~15% of the total soluble cellular protein) that comigrated with the recombinant wild-type human 15-lipoxygenase on SDS–PAGE (Figure 1, inset). This band was immunoreactive to antibodies against the recombinant human 15-lipoxygenase and was not present in the insect cell cultures that had been prepared with vectors not containing 15-lipoxygenase cDNA (data not shown).

Using anion exchange chromatography, lipoxygenase activity coeluted with a major protein peak on a Protein Pak Q8 semi-preparative FPLC column at a sodium chloride concentration of 30 mM (Figure 1). This one-step purification resulted in lipoxygenase of >90% purity, as judged by SDS–PAGE stained with Coomassie brilliant blue (Figure 1, inset). Routinely, 40–50% of the lipoxygenase activity was recovered from the column, and a 7.5-fold enrichment of the specific

Table II. Purification of 15LOX (I417V,M418V) from baculovirus–insect cell culture

Fraction	Protein (mg)	Total activity[a]	Specific activity[b]	Fold pure	Yield (%)
Lysate	40.5	652.2	0.016	–	100
Q8	2.4	288.0	0.120	7.5	44

[a]Units are expressed as nmol conjugated diene formed per minute at 4°C, based on ΔOD_{234}/min in the first 3 min of the reaction. An extinction coefficient of 23 000 M/cm was used (Gibian and Vandenberg, 1987).
[b]Specific activity is expressed as μmol conjugated diene/min/mg protein.

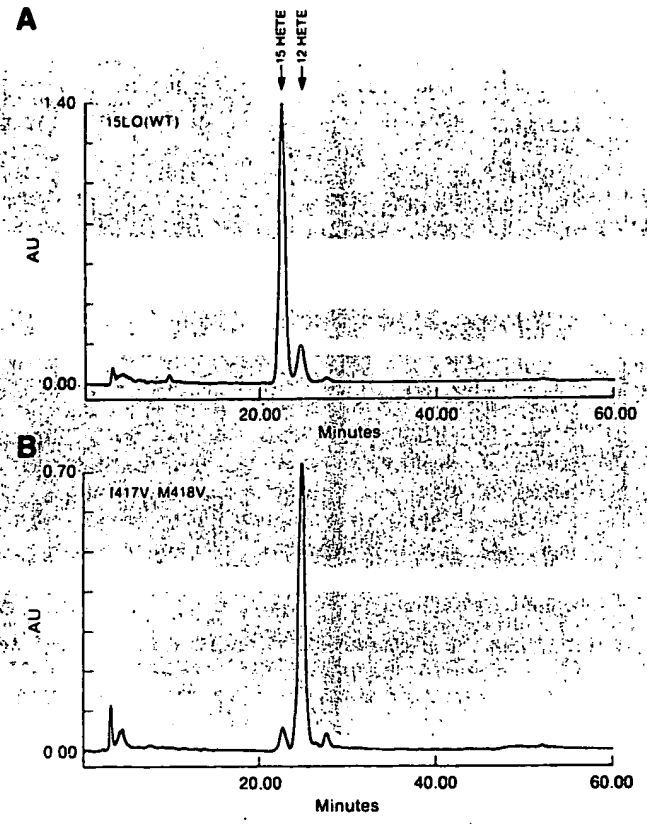

Fig. 2. Reverse-phase HPLC chromatograms (monitored at 234 nm) of the oxygenation products of purified wild-type (**A**) and mutant [15LOX (I417V,M418V)] (**B**) lipoxygenase. Assay conditions have been described previously (Sigal *et al.*, 1990). Enzyme purified by anion exchange chromatography was incubated with 300 μM arachidonic acid, the lipids were reduced with trimethyl phosphite, extracted for HPLC analysis and injected onto a reverse-phase C-18 column. The column effluent was monitored continuously at wavelengths between 200 and 300 nm on a Waters 996 photo-diode array detector (Millipore). (**B**) 15LOX (I417V,M418V) resulted in the production of a major peak absorbing at 234 nm which coelutes with authentic 12-HETE. (**A**) Wild-type 15-lipoxygenase resulted in the production of a major peak absorbing at 234 nm which coelutes with authentic 15-HETE. Coelution was confirmed by coinjection with authentic 12- and 15-HETE (data not shown).

activity was achieved (Table II). We have routinely recovered 20–30 mg of pure enzyme per liter of culture.

The 15LOX (I417V,M418V) variant expressed and purified in this way catalyzes the oxygenation of arachidonic acid to 15-HPETE and 12-HPETE in a ratio of 1:20, as shown by reverse-phase HPLC of the corresponding hydroxy fatty acids obtained after reduction by trimethyl phosphite (Figure 2B). This is the same ratio found in the bacterial cell assay and is completely the reverse of that from purified wild-type 15-lipoxygenase (Figure 2A).

Table III. A comparison of the steady-state kinetic parameters of the wild-type 15-lipoxygenase and 15LOX (I417V,M418V)

Lipoxygenase	Arachidonic acid		Linoleic acid	
	K_M (μM)	V_{max} (U/μg)[a]	K_M (μM)	V_{max} (U/μg)[a]
Wild-type	11.7 ± 0.9	0.73 ± 0.20	14.3 ± 3.9	1.5 ± 0.2
I417V,M418V	9.6 ± 1.4	0.22 ± 0.07	9.9 ± 2.6	0.16 ± 0.06

Values are means ± SEM; $n = 3$.
[a]Units are expressed as nmol conjugated diene produced per minute.

Table IV. Relative initial reaction rates of various fatty acids by the wild-type and 15-LOX (I417V,M418V) lipoxygenases

Substrate	Wild-type (%)	I417V,M418V (%)
Arachidonic acid	100	100
Eicosa-11Z,14Z,17Z-trienoic acid	140.0	8.3
Eicosa-5Z,8Z,11Z-trienoic acid	40	275
Eicosa-8Z,11Z,14Z-trienoic acid	400	150
Eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid	70	92
Docosa-4Z,7Z,10Z,13Z,16Z,19Z-hexaenoic acid	100	96

The steady-state kinetics of the oxygenation of arachidonic acid and linoleic acid catalyzed by the 15LOX (I417V,M418V) were compared with the wild-type enzyme. Using arachidonic acid as a substrate, the wild-type and mutant enzymes had very similar apparent K_Ms. The V_{max} of the mutant enzyme is reduced by a factor of 3 (Table III). Using linoleic acid as substrate, the apparent K_Ms for the mutant and wild-type enzymes are similar; however, the mutant enzyme has a V_{max} 10 times lower than the wild-type enzyme, as expected for an enzyme that predominantly catalyzes the oxygenation of an ω-9 carbon.

The activity of 15LOX (I417V,M418V) on a variety of fatty acids was compared with the wild-type 15-lipoxygenase to analyze the mutant's requirements for the position of a bis-allylic methylene (Table IV). Each enzyme was allowed to react in a quartz cuvette at 4°C with the various fatty acids at 50 μM. The change in absorbance at 234 nm was monitored continuously for 3 min. The initial reaction rates were determined as the change in optical density per minute. The initial rates were compared with that of arachidonic acid, presented as 100%, which corresponded to 0.45 nmol/min for both the wild-type and mutant enzymes. Fatty acids which contained only three unsaturated positions were slightly better as substrates than arachidonic acid. The wild-type enzyme prefers substrates which contain a bis-allylic methylene at the ω-8 position (arachidonic acid, eicosa-11,14,17-trienoic acid, eicosa-8,11,14-trienoic acid, eicosa-5,8,11,14,17-pentaenoic acid). In contrast, the 15LOX (I417V,M418V) prefers substrates which contain a bis-allylic methylene at the ω-11 position (arachidonic acid, eicosa-8,11,14-trienoic acid, eicosa-5,8,11-trienoic acid, eicosa-5,8,11,14,17-pentaenoic acid) and rejects eicosa-11Z,14Z,17Z-trienoic acid which has a bis-allylic methylene at the ω-8 position.

Discussion

Defining the role that specific amino acids in an enzyme play in substrate recognition is of prime importance in understanding

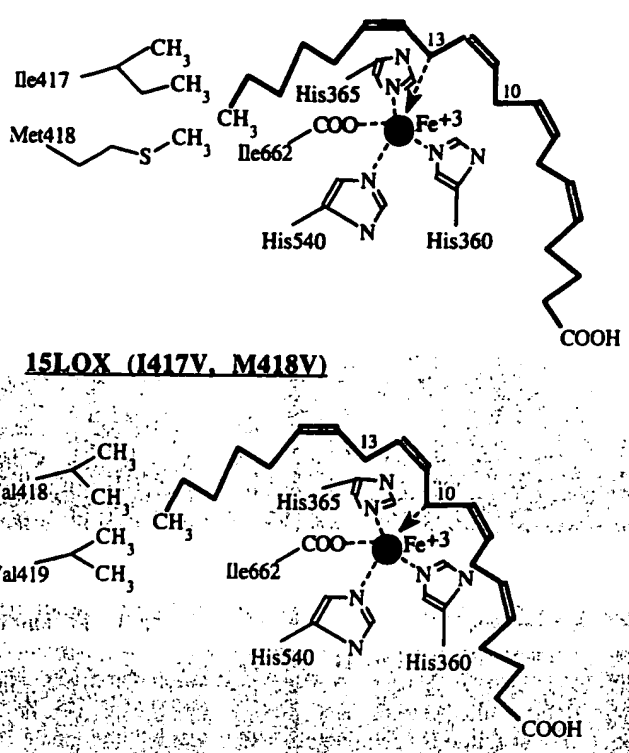

Fig. 3. Models depicting the difference in substrate positioning by 12- and 15-lipoxygenases. Arachidonic acid is shown as the substrate. The iron binding ligands are inferred from the 3-D structure of the soybean enzyme (Boyington *et al.*, 1993; Minor *et al.*, 1993). (**A**) Amino acids at positions 417 and 418 of human 15-lipoxygenase define a region in the substrate binding pocket which enables the substrate to bind in the proper catalytic register for 15-lipoxygenation. For 15-lipoxygenation, hydrogen abstraction occurs at bis-allylic methylene carbon-13 (arrow). (**B**) Valines at positions 417 and 418 enable the substrate to bind in the proper catalytic register for 12-lipoxygenation. For 12-lipoxygenation, hydrogen abstraction occurs at bis-allylic methylene carbon-10 (arrow).

enzyme function. By altering the specificity of an enzyme with amino acid substitutions at key positions, the primary determinants of specificity can be located. An analysis of the primary structure of the family of lipoxygenases permitted us to focus our attention on four conserved differences that could be critical in distinguishing a 12-lipoxygenase from a 15-lipoxygenase. Mutational analyses showed that amino acid 418 is crucial in determining the positional specificity of 15-lipoxygenase (Sloane *et al.*, 1991). The 3-D structure of the 15-lipoxygenase from soybean seeds has been reported (Boyington *et al.*, 1993). By sequence alignment, the amino acid in the primary structure of the soybean enzyme which corresponds to methionine 418, phenylalanine 557, lies in a solvent-accessible cavity of the protein, suggesting that this cavity may be the fatty acid binding cavity. Since the structure was solved in the absence of substrate or substrate analogs, it is difficult to draw any conclusions about the binding of the substrate in the enzyme from the structure alone. However, the mutagenesis data presented here demonstrate that the assignment of this region as the fatty acid binding cavity is reasonable. Furthermore, the distance between the phenylalanine 557 and the iron (14 Å; L.M.Amzel, personal communication) is close to the length of the fatty acid chain from the methyl terminus to the ω-8 carbon.

The results presented in this study are consistent with a model in which amino acids at positions 417 and 418 of 15-lipoxygenase define a structure that positions the substrate relative to the reaction center of the enzyme (Figure 3). When either the isoleucine at position 417 or the methionine at position 418 were replaced with valine, the resulting activity yielded equal 12- and 15-HETE. This implies a partial shift of the substrate from a position where the reaction center is aligned optimally for 15-lipoxygenation to a position where it is aligned for 12-lipoxygenation. The replacement of both isoleucine 417 and methionine 418 with valines results in activity which is similar to that of wild-type 12-lipoxygenase, implying a complete shift in the binding of the substrate to a position where 12-lipoxygenation is preferred.

One interpretation of this result is that the amino acid side chains interact directly with the methyl terminus of the fatty acid substrate. The smaller valines define a deeper pocket, thus allowing the substrate to bind deeper into the enzyme and aligning the reaction center of the enzyme with carbon-10 of arachidonic acid (the site of hydrogen abstraction for 12-lipoxygenation). Two different mutations were carried out to test this interpretation.

Firstly, by replacing methionine 418 with asparagine, we introduced a polar side chain at this position. If the hydrophobicity of this side chain was important, asparagine should have a deleterious effect on the enzyme's activity. However, this mutation had activity similar to that of the 15LOX (M418V). Valine and asparagine have a similar bulk (Ponder and Richards, 1987); therefore, the side-chain bulk appears to be more important than its chemical characteristics at this position. Further evidence in support of this conclusion comes from the activity of the 15LOX (M418K) mutation. Methionine and lysine both occupy 171 Å^3 (Ponder and Richards, 1987); however, methionine is hydrophobic and lysine is charged. The 15LOX (M418K) variant showed no change in its activity, implying that the side chain is not in contact with the substrate but defines the positional specificity of the enzyme in a less direct way.

Secondly, when the methionine was replaced with an isoleucine, the change in the activity of the enzyme was similar to changing the methionine to a valine (i.e. an enzyme which performs equal 12- and 15-lipoxygenation was produced). However, when a methionine to leucine change was performed, the enzyme had activity that remained similar to that of wild-type 15-lipoxygenase. Therefore, a change as subtle as moving a methyl group from the β to the γ carbon of this amino acid can have a dramatic effect on the enzyme's activity. The introduction of threonine at this position affected the enzyme's positional specificity, but not as severely as a valine. The hydroxyl group of the threonine can potentially make a hydrogen bond that is not possible with methionine or valine at this site. This could alter a key structural feature of the positional specificity region in a way that is different from any of the other amino acid changes.

In this study, we found no effect on the K_M of the 15LOX (I417V,M418V) enzyme for arachidonic acid, and very little change in the V_{max} of the reaction. This implies that there was no change in a key energetic interaction between the enzyme and the substrate. The important difference between the wild-type and mutant 15-lipoxygenase appears to be that the mutant allows the substrate to bind 'deeper', aligning the substrate in the active site to favor 12-lipoxygenation. Using linoleic acid as a substrate, the mutant has a similar K_M; however, the V_{max}

is 10 times less. This result is also consistent with the model (Figure 3). When linoleic acid is bound 'deeper' into the enzyme, carbon-11 (the site of hydrogen abstraction, the first rate-limiting step of the reaction; Hamberg and Samuelsson, 1967) is moved out of an optimal position, resulting in a decreased reaction rate. Thus, a prediction of the model is that 15LOX (I417,M418V) requires a bis-allylic methylene near the ω-11 position of the substrate, whereas wild-type 15-lipoxygenase requires a bis-allylic methylene near the ω-8 position.

This prediction was tested by assaying the activity of wild-type and 15LOX (I417V,M418V) on a variety of unsaturated fatty acids (Table IV). Comparing the activities of the enzymes on eicosa-11,14,17-trienoic acid with the activities on eicosa-5,8,11-trienoic acid shows that the wild-type enzyme is more active on the substrate with a bis-allylic methylene at the ω-8 position (eicosa-11,14,17-trienoic acid), and the mutant is more active on the substrate with a bis-allylic methylene at the ω-11 position (eicosa-5,8,11-trienoic acid). Both enzymes are more active on eicosa-8,11,14-trienoic acid than on arachidonic acid, as has been reported previously for rabbit 15-lipoxygenase (Kuhn *et al.*, 1990b). The enzymes are equally active on the less saturated fatty acids (arachidonic acid, eicosa-5,8,11,14,17-pentaenoic acid and docosa-4,7,10,13,16,19-hexaenoic acid), all of which contain bis-allylic methylenes at both the ω-8 and ω-11 positions.

Two catalytically and immunologically distinct types of 12-lipoxygenase have been characterized (Takahashi *et al.*, 1988), the 'platelet-type' which has a narrow substrate range and a strict requirement for an ω-9 fatty acid, and the 'leukocyte-type' which has a broader substrate range and exhibits dual positional specificity, introducing oxygen at the ω-6 position as well as at the ω-9 position (Hada *et al.*, 1991). The 'leukocyte-type' 12-lipoxygenase also catalyzes the oxygenation of fatty acids esterified to phospholipids (Takahashi *et al.*, 1993), consistent with the possibility that this enzyme interacts predominantly with the methyl terminus of the substrate, as proposed for 15-lipoxygenase. The reduced activity of 15LOX (I417V,M418V) on linoleic acid, as well as its dual positional specificity on arachidonic acid, demonstrate that this variant is more similar to the 'leukocyte-type' 12-lipoxygenase. Furthermore, the activity of the 15LOX (I417V,M418V) on the variety of fatty acids we tested is very similar to those reported previously for the 12-lipoxygenase from bovine leukocytes (Takahashi *et al.*, 1988). However, the bovine leukocyte 12-lipoxygenase did not show the increased activity on eicosa-8Z,11Z,14Z-trienoic acid, a difference that does not change the interpretation of our data.

Previously, Chen and Funk (1993) mutated the human platelet 12-lipoxygenase to introduce 15-lipoxygenase sequences and expressed the variant enzymes in a transient transfection of human embryonal kidney cells. These authors found that a single methionine to valine change had no effect on the positional specificity of the enzyme; however, a triple mutation (K416Q,A417I,V418M) resulted in an enzyme which performed 15-lipoxygenation with a 12-HETE:15-HETE ratio of ~4:1. Further replacement of all amino acids between positions 398 and 429 of the human platelet 12-lipoxygenase with those found in 15-lipoxygenase resulted in an enzyme which performed 12:15-lipoxygenation with a ratio of 1:2. During the course of this study we generated two different mutations of the 12-lipoxygenase from bovine tracheal epithelium (a 'leukocyte-type' enzyme) to make the converse changes of those made on 15-lipoxygenase. Neither mutant completely converted the enzyme from a 12-lipoxygenase to a 15-lipoxygenase; however, the double mutant 12LOX (V418I,V419M) did have altered positional specificity, showing a change in the 12-HETE:15-HETE ratio from 15:1 to 3:1. Thus, certain 12-lipoxygenases contain other structural elements that are key features for its positional specificity. However, the porcine leukocyte 12-lipoxygenase appears to determine its positional specificity in a way similar to the model in Figure 3, as mutations on this enzyme which converted the two valines to isoleucine and methionine have been reported to switch the positional specificity from a 12-lipoxygenase to a 15-lipoxygenase (Suzuki *et al.*, 1994).

Naturally occurring 12-lipoxygenases have been cloned from rat brains (Watanabe *et al.*, 1993) and mouse leukocytes (Chen *et al.*, 1994) which contain methionine at the same position in the sequence as 15-lipoxygenase methionine 418. Mutagenesis of this methionine, as well as neighboring amino acids, had no effect on the positional specificity of the rat 12-lipoxygenase (Watanabe and Haeggström, 1993). It is interesting to note that the ratio of 12:15-lipoxygenase products synthesized by the rat brain lipoxygenase is 6:1 and for the mouse leukocyte enzyme is 3:1. Thus, these two enzymes with methionine at position 418 have a product profile that is shifted towards a 15-lipoxygenase, a result that is similar to the mutagenesis on the bovine 12-lipoxygenase.

We and others (Chen and Funk, 1993) have been unable to change the positional specificity to that of a 5-lipoxygenase by mutagenesis. Replacement of the amino acids at positions 416–419 of 15-lipoxygenase with those found in 5-lipoxygenase resulted in the expression of a protein with no detectable activity. This demonstrates that there are other structural features of 5-lipoxygenase that account for its positional specificity.

Taken together, these results imply that the amino acids at positions 417 and 418 define the positional specificity region of 15-lipoxygenase indirectly, rather than by a direct side chain–substrate interaction. Changing the side-chain bulk at position 418 leads to a shift in activity towards 12-lipoxygenation. No changes in the binding energetics of the substrate were detected. Therefore, rather than altering a direct interaction between the substrate and the enzyme, the 15LOX (I417V,M418V) mutant allows the binding of the arachidonic acid substrate in a different register, favoring 12-lipoxygenation. This effect is dependent more on the amino acid side-chain bulk and geometry than on its chemical characteristics. The insensitivity of this structure to either hydrophobicity or charge implies that the effect of this amino acid is mediated through conformational or configurational parameters. Further dissection of the substrate binding interactions of 15-lipoxygenase, coupled with X-ray crystallography or modeling with the soybean structure, will aid our understanding of how amino acids at positions 417 and 418 define the positional specificity of the enzyme; research will also demonstrate what roles other amino acids in the substrate binding pocket play in the structural basis of lipoxygenase function.

Acknowledgements

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      421 nqlnsaktya trtilflred gtlkpvaiel slphsagdls aavsqvvlpa kegvestiwl
      481 lakayvivnd scyhqlmshw lnthaamepf viathrhlsv lhpiyklltp hyrnnmnina
      541 larqslinan giiettflps kysvemssav yknwvftdqa lpadlikrgv aikdpstphg
      601 vrlliedypy aadgleiwaa iktwvqeyvp lyyardddvk ndselqhwwk eavekghgdl
      661 kdkpwwpklq tledlvevcl iiiwiasalh aavnfgqypy gglimnrpta srrllpekgt
      721 peyeeminnh ekaylrtits klptlislsv ieilsthasd evylgqrdnp hwtsdskalq
      781 afqkfgnklk eieeklvrrn ndpslqgnrl gpvqlpytll ypsseegltf rgipnsisi
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Structure conservation in lipoxygenases: structural analysis of soybean lipoxygenase-1 and modeling of human lipoxygenases.

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Lipoxygenases are a class of non-heme iron dioxygenases which catalyze the hydroperoxidation of fatty acids for the biosynthesis of leukotrienes and lipoxins. The structure of the 839-residue soybean lipoxygenase-1 was used as a template to model human 5-, 12-, and 15-lipoxygenases. A distance-based algorithm for placing side chains in a low homology environment (only the four iron ligands were fixed during side chain placement) was devised. Twenty-six of the 56 conserved lipoxygenase residues were grouped in four distinct regions of the enzyme. These regions were analyzed to discern whether the side chain interactions could be duplicated in the models or whether alternate conformers should be considered. The effects of site directed mutagenesis variants were rationalized using the models of the human lipoxygenases. In particular, variants which shifted positional specificity between 12- and 15-lipoxygenase activity were analyzed. Analysis of active site residues produced a model which accounts for observed lipoxygenase positional specificity and stereospecificity.

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5-Lipoxygenase from potato tubers. Improved purification and physicochemical characteristics

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Key words: 5-Lipoxygenase; 11-Lipoxygenase; ESR; Leukotriene A_4; Hydroperoxyoctadecadienoic acid; Hydroperoxyeicosatetraenoic acid; (Potato tuber)

Potato tubers are shown to contain at least three lipoxygenase isoenzymes. A very efficient extraction of lipoxygenase activity is obtained when a non-ionic detergent (0.1% Brij 99) is added to the homogenization buffer. The major isoenzyme, L_1, has been purified in an almost homogeneous form with a good yield (18%) and a high specific activity (140–160 units/mg). It is efficiently stabilized by glycerol (20%, v/v). The purified L_1 isoenzyme is slightly contaminated by an 11-lipoxygenase, both having very close p*I* values (4.94 and 4.99, respectively). L_1 is a monomeric protein of M_r 92000 containing one iron atom per molecule. The native enzyme is in a pseudo-axial high-spin ferric state as indicated by EPR. Acting on linoleic acid, L_1 forms 9-hydroperoxyoctadecadienoic acid (9-HPOD) almost exclusively. With arachidonic acid, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) is the major product (70–75%) beside small amounts of 8(*S*)- and 9-HPETE. Due to the contaminating activity, 11-HPETE (15%) is also present. Formation of both 8(*S*)-HPETE and leukotriene A_4 hydrolysis products accounts for the intrinsic 8-lipoxygenase activity of the L_1 isoenzyme.

Introduction

Lipoxygenases (linoleate/oxygen oxidoreductase, EC 1.13.11.12) catalyze the stereospecific insertion of dioxygen into polyunsaturated fatty acids containing at least one 1,4-(*Z*,*Z*)-pentadienyl unit [1,35,36]. Acting on arachidonic acid, 5-lipoxygenase leads to 5(*S*)-HPETE, which is the precursor of biologically active leukotrienes [2].

As part of a general project aiming at the understanding of the reaction mechanism of these enzymes, we needed a convenient source to get a 5-lipoxygenase easily and in reasonable yield. Several mammalian 5-lipoxygenases have recently been purified and characterized [3–8], but, beside their low stability, they are not available in large amount. Among vegetal lipoxygenases the potato enzyme is one of the few showing a 5-lipoxygenase activity [9–12]. A pure preparation is able to catalyze the formation of the unstable leukotriene A_4,

Abbreviations: Brij 99, $HO-(CH_2-CH_2O)_{20}-(CH_2)_8-CH=CH-(CH_2)_7-CH_3$; L_1, major isoenzyme of potato 5-lipoxygenases; HPETE, hydroperoxyeicosatetraenoic acid; HPOD, hydroperoxyoctadecadienoic acid; Detapac, diethylenetriaminepentaacetic acid; NDGA, nordihydroguaiaretic acid; ABPH, acetonylacetone bisphenylhydrazone; BPH, benzaldehyde phenylhydrazone.

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epoxide from 5(*S*)-HPETE, which is the primary and major product of the arachidonic acid reaction [10].

In this paper, we present a short and reproducible procedure to get the major isoenzyme of potato lipoxygenases in an almost homogeneous forms with a yield and specific activity higher than those previously reported. The reactions with linoleic and arachidonic acids have been studied in detail, showing in the latter case an intrinsic 8-lipoxygenase activity likely to account for the leukotriene A_4 hydrolysis products detected. Finally, EPR data support an iron ferric state for the native purified enzyme.

Materials and Methods

Tween 20, sodium acetate and sodium bisulfite were reagent grade from Merck. Brij detergents (polyoxyethylene ethers), diethylenetriamine pentaacetic acid (Detapac), nordihydroguaiaretic acid (NDGA), arachidonic acid (99%) and linoleic acid (99%) were from Sigma. 3-Amino,1-(3-trifluoromethyl)-2-pyrazoline-HCl (BW 755C) was a gift from Rhone-Poulenc, France. Acetonylacetone bisphenylhydrazone (ABPH) and benzaldehyde phenylhydrazone (BPH) were prepared according to published procedures [23] and had correct elemental analysis and spectroscopic characteristics. DEAE-Trisacryl gel was purchased from IBF. Ultrapure water was obtained through a reverse osmosis system (Elga, Millipore).

Enzyme preparations

L_1 soybean lipoxygenase (160–200 U/mg) was purified as described in Ref. 31. L_1 potato lipoxygenase was purified at 4°C as described in the text. The following cultivars were screened for 5-lipoxygenase activity: Bintje, Dani, Daresa, Desiree, Kaptah-Vendel, Kennebec, Lizen and Norchip. They were purchased from INRA, Station d'Amelioration de la pomme de terre et des plantes à bulbe, BP 5, 29207 Landerneau cedex, France. The purification of the L_1 5-lipoxygenase herein described was done with the Bintje variety.

Assays

Lipoxygenase activity was measured at 23°C by monitoring the increase in absorbance at 234 nm of the forming hydroperoxides ($\epsilon = 25\,000$ $M^{-1} \cdot cm^{-1}$) or, in some instance, the oxygen uptake using a Clark oxygen-electrode with a Gilson oxygraph assuming a 240 μM O_2 concentration in air-saturated buffer at 23°C. Both methods gave identical results. Reaction buffer was 0.2 M sodium acetate containing 0.5 mM Detapac and 0.001% Tween 20 (w/v) (pH 5.5). One activity unit was defined as the production of 1 μmol HPOD per min from 150 μM linoleic acid. Protein concentrations was routinely assayed by measuring the absorbance at 280 nm ($\epsilon = 140\,000$ $M^{-1} \cdot cm^{-1}$). For the purified samples of lipoxygenases, a good agreement with the results of the protein assay described by Lowry et al. [34] was found. Before each set of experiments, fresh solutions of the desired fatty acid were prepared as follows: purification of the commercial high-purity acid on Sep-Pak straight-phase cartridge (Waters) yielded a peroxide-free solution in hexane/ether (95 : 5). Thorough evaporation of solvents followed by addition of Tween 20 (1 : 1, w/w, vs. substrate) and solubilization in degassed water gave a turbid solution which became clear upon addition of few drops of 2 M NH_4OH. The solution was then adjusted to the desired concentration by addition of degassed water. A reverse-phase HPLC analysis of this solution showed a baseline recording in the hydroperoxide region.

Leukotriene A_4 synthetase activity was assayed at 23°C by anaerobic incubation of 5-HPETE with the enzyme as described by Bryant et al. [20]. After completion of the reaction (monitored by ultraviolet spectroscopy) the reaction mixture was brought to pH 9.00 with dilute sodium hydroxide and the unreacted 5-HPETE reduced by $NaBH_4$. After cautious acidification with 2 M citrate (pH 4.00) and ether extraction, the crude reaction mixture was esterified with an excess of ethereal diazomethane. The dihydroxymethyl esters derivatives were analyzed on a semi-preparative straight-phase HPLC column as indicated below.

Determination of the absolute configuration of 8-HPETE

Pure 8-HETE was obtained through straight-phase HPLC purification and standard $NaBH_4$ reduction of the hydroperoxide. The ammonium salt (3 μg) of this product was reacted at pH 9 in

0.5 ml 20 mM borate buffer with 1 μg of highly purified L_1 soybean lipoxygenase. The conversion of the diene chromophore into the conjugated triene (λ = 259, 269, 280 nm) was complete within 5 min at room temperature. After reduction and elution from a C18 Sep-Pak cartridge the reaction mixture was analyzed by reverse-phase HPLC (isocratic elution, H_2O/ MeOH/ CF_3COOH/ Et_3N (200 : 800 : 1 : 0.5) at a flow rate of 0.8 ml/min). Two compounds were found in a 91 : 9 ratio. They were identified as 8(*S*),15(*S*)-diHETE (retention time, 7.33 min) and 8(*R*),15(*S*)-diHETE (retention time, 8.02 min), respectively, by coelution with authentic samples. The same reaction performed on racemic 8-HETE obtained by arachidonate autoxidation yielded the same two compounds in a 49 : 51 ratio.

Analytical methods

SDS-polyacrylamide gel electrophoresis was performed at 10 °C with 10% bisacrylamide gels in phosphate buffer containing 0.1% SDS (pH 8.3) in the presence or absence of 2-mercaptoethanol. The molecular weight protein standards (20 000 × 94 000) were from Pharmacia. A 2103 Ultraphor LKB system was used. The current was set at 8 mA per tube. The migration lasted for 8 h. Samples contained 25–100 μg of proteins. The gels were stained for protein with Coomassie brillant blue R-250 and diffusion destained in 10% acetic acid. They were scanned for protein density on a Sebia system 2 densitometer.

Isoelectric focusing was run on an electrofocusing unit 2217 Ultraphor equipped with a 2103 power supply and multitemperature 2209 from LKB. About 100 μg of protein were deposited on the plate (ampholines-polyacrylamide gel plate, pH = 4–6.5). The gel was stained, destained and scanned for protein density as described above.

Atomic absorption spectrometry was performed on a Pye Unicam FP9 Philips apparatus equipped with a hollow electrode specific for iron (λ = 248.3 nm). Standardization was done with calibrated $FeCl_3$ solutions obtained by dissolving high-purity iron in concentrated HCl.

HPLC reverse-phase chromatography was run on a Kontron liquid chromatograph system equipped with a 200 programmer. A 5 μm C_{18} Spherisorb column was used for isocratic elution (HPETEs detection at 237 nm). Acetonitrile (65%) in H_2O containing 0.1% acetic acid was used as eluent (flow, 1.5 ml/min.). DiHETEs were purified on a semi-preparative μPorasil column (Waters). The mobile phase was composed of solvent A (hexane/0.1% acetic acid) and solvent B (hexane/1% isopropanol/0.1% acetic acid) delivered with a linear gradient at 4 ml/min. The gradient started at 20% solvent B, going up to 100% solvent B in 30 min. The effluents were monitored at 250 and 280 nm. The 280 nm-absorbing fractions were collected, evaporated to dryness under nitrogen and submitted to GC-MS analysis after derivatization as previously described [33].

Electron-impact mass spectra analyses by GC-MS were performed on a LKB 2091 mass spectrometer interfaced to a PDP 11 data system. Mass spectra were recorded at 70 eV. Multiple ion detection was used for monitoring the major fragments of dihydroxylated compounds. A specifically designed capillary column was used in most of the GC-MS studies. This column showed very little, if any, adsorption of 5- and 8-hydroxylated derivatives, as generally occurs with most of commercially available columns [33].

Ultraviolet spectra and kinetic studies were performed on a Uvikon 920 Kontron spectrometer.

EPR spectra were recorded at X-band frequency with a Bruker ER 200 spectrometer at 4–15 K using an Oxford Instruments continuous flow cryostat, Hall probe and a Hewlett-Packard frequency meter (modulation frequency, 100 KHz; microwave power; 20 mW; field modulation intensity; 3.2 Gpp; gain, $2.5 \cdot 10^5$).

Results

Purification procedure

The purification herein described is mainly based on the procedure of Sekiya et al. [12], with the important difference of adding a non-ionic detergent (0.1%) to the extraction buffer.

A comparison of the effectiveness of several detergents is shown in Table I. From these data, it appears that the addition of millimolar concentration of Brij detergents to the homogenization buffer, 0.1 M sodium acetate/2 mM sodium bisulfite/0.1 mM Detapac (pH 4.5), allows a much

TABLE I

EXTRACTION OF LIPOXYGENASE ACTIVITY FROM HOMOGENATE OF POTATO TUBERS. EFFECTS OF NON-IONIC DETERGENTS

Diced potato tubers (100 g) in 100 ml buffer (0.1 M sodium acetate/2 mM sodium bisulfite (pH = 4.5)) and the indicated concentration of detergent were homogenized in a Waring Blendor (25000 rpm) at 4° C (2 × 1 min.), filtered on gauze and centrifuged at 20000 × *g* for 20 min. The supernatant was used as source of the lipoxygenase. For extracted proteins, the estimated protein concentration was calculated from absorbance at 280 nm, assuming an $\epsilon = 140000\ M^{-1} \cdot cm^{-1}$ [31]. 1 activity unit is defined as the formation of 1 μmol HPOD per min, calculated from the increase of absorbance at 234 nm ($\epsilon = 25000\ M^{-1} \cdot cm^{-1}$) of the forming hydroperoxides. Linoleic acid is 150 μM. Assay buffer, 0.2 M sodium acetate/0.1 mM Detapac (pH 5.5). IC_{50}, concentration of detergent which gives 50% inhibition of linoleic acid (150 μM) hydroperoxidation with 0.2 unit of lipoxygenase.

Exp. No.	Detergent (mM)	Extracted proteins (mg/ml)	Spec. act. (units/mg)	IC_{50} (μM)
A_1	–	7.7	0.56	
A_2	Tween 20 (2.8)	9.8	1.10	500
A_3	Brij 56 (2.8)	11.8	1.80	180
A_4	Brij 96 (2.8)	10.7	2.90	160
B_1	–	6.75	0.50	
B_2	Brij 96 (0.15)	6.90	0.92	
B_3	Brij 96 (1.5)	7.70	1.80	
B_4	Brij 96 (7.5)	8.15	2.00	
C_1	–	5.2	0.56	
C_2	Brij 99 (1.15)	6.8	2.40	120 ($K_i = 47$)

better recovery of lipoxygenase activity. This effect levels off at about 2 mM detergent concentration. Brij detergents inhibit the enzyme, as appears from the IC_{50} values given in Table I. Despite a rather efficient competitive inhibition ($K_i = 47$ μM), Brij 99 was selected because of the smaller amount necessary to give a purer protein. It was also found to protect the enzyme against inactivation during the purification and storage (about 40% loss of activity after 6 months at −80° C). However, in that respect, glycerol (20%, v/v), which is not at all inhibitory, was found to stabilize the enzyme better (less than 10% loss of activity after 6 months at −80° C).

The centrifuged homogenate (20000 × *g*, for 20 min) was directly dialyzed overnight against 3 vol. of 20 mM Tris-acetate buffer containing 0.1 mM Detapac as trace metal chelator (pH 7.4). After removal of precipitated inactive proteins, the lipoxygenase activity was recovered in the fraction between 30 and 50% ammonium sulfate saturation.

The pellets were dissolved into a minimum of Tris buffer and dialyzed versus 2 × 1 l of this buffer.

The clear colorless supernatant was introduced on top of a DEAE-Trisacryl column (40 × 3 cm) previously equilibrated with the above-mentioned Tris buffer. The column was washed with 2 bed vol. and the proteins were eluted with a linear gradient (0–0.18 M) of sodium acetate in Tris buffer (pH 7.4).

Under strictly non-saturating conditions (typically less than 1 g of protein) the lipoxygenase activity was found in three separate peaks (Fig. 1), eluting at approx. 0.10, 0.12 and 0.15 M sodium acetate, respectively. The corresponding isoenzymes are later called L_1, L_2 and L_3. The active fractions of the L_1 major isoenzyme were pooled and dialyzed against the above-mentioned Tris buffer containing 20% glycerol (v/v) for 20 h (1 × 3 l). Omitting the addition of glycerol at this step results in a significant loss of activity during dialysis.

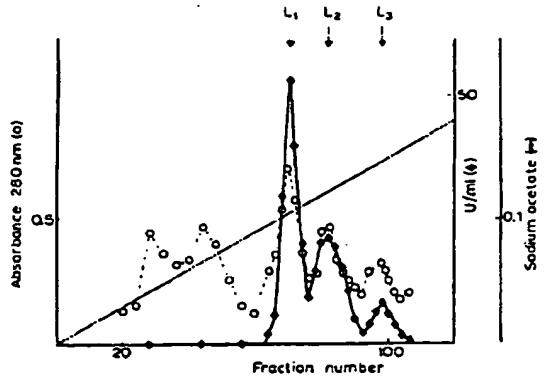

Fig. 1. DEAE-Trisacryl column profile of a dialyzed, fractionated potato homogenate. The column is washed with 2 bed vol. of buffer/20 mM Tris-sodium acetate/0.1 mM Detapac (pH 7.4). Thereafter, a linear gradient of sodium acetate is applied (0–0.18 M). The flow rate is set at 120 ml/h and the effluent collected in 20-ml fractions. The lipoxygenase activity is defined as in Table I.

The dialysate was then charged on a second DEAE-Trisacryl column (20 × 3 cm) equilibrated with the Tris-glycerol buffer. The column was washed with 2 bed vol. and a linear gradient (0–0.13 M) of sodium acetate in buffer was then applied. The lipoxygenase activity was recovered at 0.10 M sodium acetate in a single peak coinciding with one protein peak only (not shown). The active fractions are conveniently stored at −80 °C, with a minimal loss of activity which is not altered by freeze-thawing cycles. The purification procedure is summarized in Table II. The major potato lipoxygenase isoenzyme (L_1) was purified about 50-fold with a specific activity of 140–160 units/mg. Starting with 250 g of tubers, about 20 mg of the L_1 5-lipoxygenase were obtained.

Properties of the purified potato lipoxygenase

Physical characteristics. Fig. 2(A) shows the densitogram of the enzyme SDS disc-gel electrophoresis on 10% polyacrylamide gels in the presence of marker proteins. Only one protein is found at M_r 92000, but the peak appears slightly distorted on the low-molecular-weight side, suggesting the presence of another protein having a very close molecular weight. A similar pattern is obtained under SDS non-reductive conditions as well as without SDS. Activity-staining showed in the latter case that lipoxygenase activity was coincident with the protein band (not shown).

TABLE II

PURIFICATION OF L_1 5-LIPOXYGENASE FROM POTATO TUBERS

For details of protein and total activity measurements, see Table I.

Step of purification	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Homogenization	7500	20000	2.7	100
25–40% ammonium sulfate saturation and dialysis	2210	17100	7.7	85.5
DEAE ion-exchange chromatography				
L_1	130	7400	56.9	37.0
L_2	104	3200	30.7	
L_3	70	2300	32.8	
Dialysis	130	5600	43.0	28.0
DEAE ion-exchange chromatography	25	3600	147.0	18.0

The procedure described above has also been utilized to purify the lipoxygenase from potatoes extracted without detergent. This gives basically the same purification factor, although with a much lower yield and activity, but SDS-polyacrylamide gel electrophoresis shows that, besides the band at

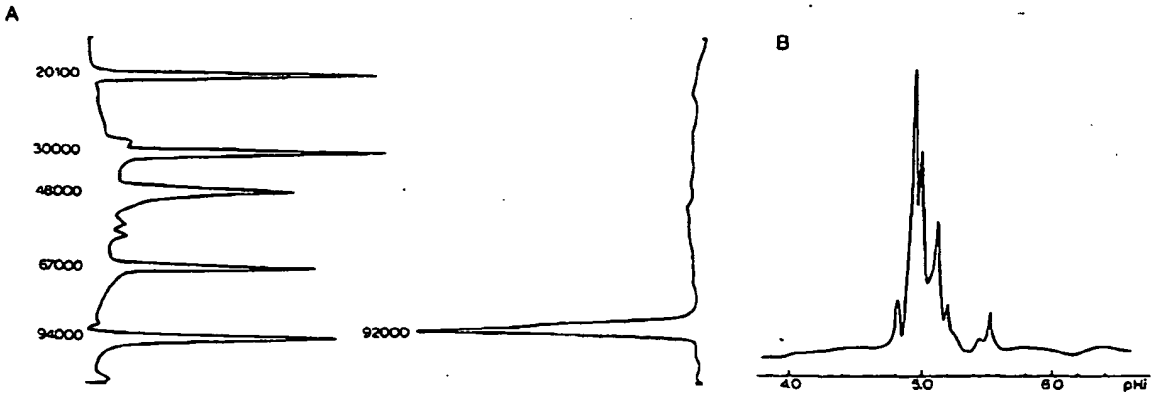

Fig. 2. (A) Densitogram of the SDS-disc gel electrophoresis of the L_1 5-lipoxygenase. About 25 μg protein were deposited and stained with Coomassie brillant blue R-250. Protein markers (Pharmacia) are as follows (molecular weight in parentheses): phosphorylase B (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100). (B) Densitogram of the isoelectric focusing of the L_1 5-lipoxygenase on Ampholines (pH 4.00–6.50) (Ampholine-PAG plate). About 100 μg protein were deposited.

92 000, major protein contaminants are present at M_r 66 000 and 41 000 (not shown).

The estimated M_r found for L_1 5-lipoxygenase is in a reasonable agreement with those given in the literature [10], but definitely lower than that of the L_1 isoenzyme from soybeans (M_r 98 000) [11].

When the purified L_1 is submitted to analytical isoelectric focusing on ampholines (pH 4–6.5), it is split into two bands with very close pI values (4.94 and 4.99) in an area ratio of about 65 : 35, both exhibiting lipoxygenase activity. A minor and inactive band is also present at higher pI (Fig. 2(B)).

The L_1 isoenzyme contains 0.95 ± 0.18 iron atom per molecule, as determined by atomic absorption spectrometry. (In the same conditions we found 0.97 ± 0.10 iron atom per molecule for several purified samples of L_1 soybean lipoxygenases from different varieties.) Interestingly, atomic absorption analysis of a dialyzed 'aged' sample stored at $-80\,^{\circ}C$ without glycerol and which had lost about 40% of its original activity, gave only 0.52 ± 0.1 iron atom per molecule, suggesting that the observed slow inactivation might be partly due to a loss of essential iron.

At variance with lipoxygenases from soybeans [13] and peas [14], native potato L_1 5-lipoxygenase exhibits an EPR spectrum which is characteristic of a high-spin Fe(III) state in a pseudo axial environment, with major signals at $g = 6.38$ and 5.9 (shoulder). (A small rhombic Fe(III) signal is also present at $g = 4.3$.) Upon addition of one or several equivalents of 9-HPOD, the low-field signals merge at $g = 6.25$, with no increase in the intensity of the overall signal (Fig. 3). Accordingly, a concentrated sample of native potato L_1 5-lipoxygenase shows the characteristic yellow colour observed with the ferric form of the L_1 soybean enzyme.

In agreement with a previous report from Pinsky et al. [11], the present isoenzymes L_1 (pure) and L_2 (partially purified) show maximal activity at pH 5.5 (Fig. 4). Potato isoenzymes are almost inactive at pH 7.5 and down at pH 3.5. Our results can be reconciled with those of Shimizu et al. [10], who found an optimal pH of 6.3 if one takes into account the report from Berkeley and Galliard [15], who demonstrated that the presence of Tween 20 used for emulsifying the fatty acid

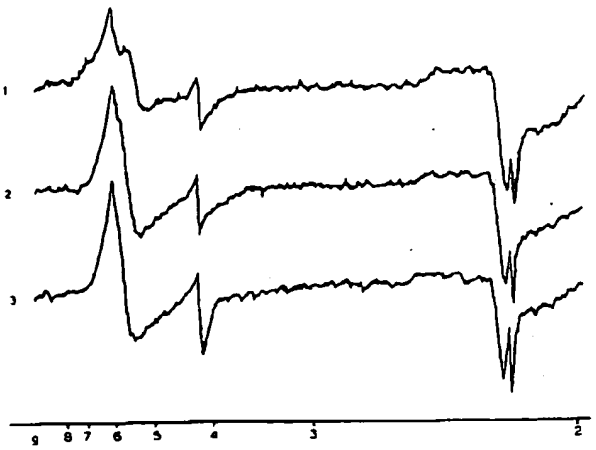

Fig. 3. EPR spectrum of the L_1 5-lipoxygenase. Trace 1, native enzyme (170 μM); trace 2, as in 1, with addition of 9-HPOD (170 μM) in 2-propanol (less than 2%, v/v); trace 3, as in 1, with 9-HPOD (680 μM) in 2-propanol.

substrates moves the optimum pH from 6.3 to 5.5, with slight broadening.

Reactions of L_1 5-lipoxygenase with linoleic and arachidonic acids. Linoleic acid is one of the best substrates of potato lipoxygenase [16]. The observed activity depends on the physical form of the solubilized substrate. The rate obtained with

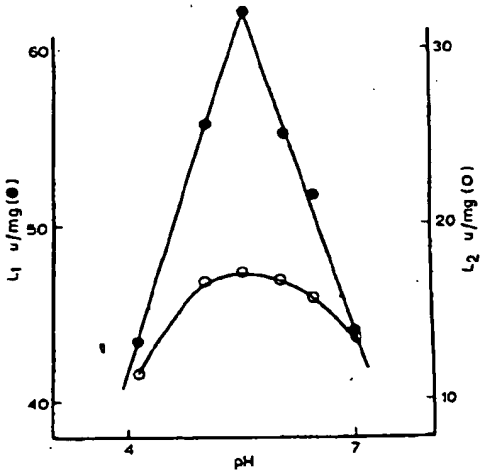

Fig. 4. Effect of pH on enzyme activity of purified L_1 and partially purified L_2 isoenzyme of potato 5-lipoxygenase. Linoleic acid (150 μM) as substrate in citrate/phosphate buffer (0.2 M).

no detergent present is about 10% of that obtained with the ammonium linoleate dispersed in 5% (w/w) Tween 20. The reaction of L_1 (0.2 units) with linoleic acid (150 μM) gives a linear production of hydroperoxides for over a 3-K_m range (see K_m determination below). However, the reaction is never quantitative (maximal conversion, 85–90%), suggesting some degree of inactivation of the enzyme during the reaction. No enzyme activation by Ca^{2+} (0.1–5 mM) could be detected.

A direct injection of the reaction mixture on reverse-phase HPLC shows only one peak. However, after diethyl ether extraction, straight-phase HPLC analysis reveals the presence of three peaks in a 2.5 : 2.5 : 95 ratio identified as (9*E*, 11*Z*)-13-HPOD, (9*Z*, 11*Z*)-13-HPOD and 9-HPOD, respectively [17]. The same experiment performed with the partially purified L_2 and L_3 isoenzymes leads to increasing amounts of the 13 isomers (15% from L_2 and 25% from L_3).

The determination of the K_m of L_1 5-lipoxygenase for linoleic acid has been performed over two orders of concentration (2–200 μM). An excellent correlation (0.9996) is obtained with $K_m = 39$ μM and $k_{cat} = 8300 \pm 400$ min^{-1}.

Arachidonic acid is a good substrate for the potato lipoxygenase. Throughout the purification, the V_{max} measured is constantly 20–25% of that found for linoleic acid. The kinetic course of the reaction shows a lag phase as well as substrate inhibition, but the main feature is the apparent autoinactivation of the enzyme. This is qualitatively demonstrated, as the reaction never goes to completion (about 75% conversion at low substrate concentration to a bare 40% at high substrate concentration). The reaction starts again with the addition of fresh enzyme only. We have determined the kinetic constant of autoinactivation according to the method of Smith and Lands [18]. When plotting instant velocity versus remaining substrate concentration, for experiments with arachidonic acid concentrations larger than K_m, a family of parallel straight lines is obtained whose slope allows k_{inact} calculation. In these conditions it was found that $k_{inact.} = 0.95 \pm 0.2$ min^{-1}. This is to be compared with the catalytic constant for the arachidonate peroxidation $k_{cat} = 1500 \pm 75$ min^{-1}. Smith and Lands [18] have reported prominent autoinactivation of the soybean enzyme (spec. act., 1.52 U/mg) when acting on arachidonic acid and other polyunsaturated fatty acids. However, in our hands, a purified sample of L_1 soybean lipoxygenase (spec. act., 212 U/mg) failed to show any autoinactivation phenomenon during its reaction with arachidonic acid (more than 95% substrate conversion). With L_1 5-lipoxygenase a reasonable fit was found (0.9987) for the determination of the K_m of the arachidonic reaction (K_m = 38 μM, $k_{cat} = 1500 \pm 75$ min^{-1}).

A reversed-phase HPLC analysis of the crude mixture from the reaction run with 80 μM arachidonic acid reveals the presence of several peaks in the HPETE region. After diethyl ether extraction, a straight-phase HPLC chromatography shows a very similar pattern. Quantification from HPLC data and from GC-MS analysis of the derivatized sample shows that 5-HPETE is the major product (70–75%), but a significant amount of 11-HPETE (15%) is also formed, beside small quantities of 8- and 9-HPETEs (Table III). No 12- or 15-HPETE were detected in this reaction. Each compound was unambiguously identified by selected ion fragments (GC-MS) and in some instances by co-elution with authentic samples (HPLC). It is noteworthy that the HPETEs distribution obtained with a detergent-free homogenate is quite similar to the one observed with the pure L_1.

We have tested eight varieties of potatoes (see Materials and Methods) and always found these HPETEs, with only small differences in their distribution. Therefore, our results differ from those of Shimizu et al. [10], who reported a 90% relative yield of 5-HPETE from the ammonium sulfate fraction of a potato homogenate obtained from a non-specified variety of tubers.

The absolute configuration of 8-HPETE has been determined as follows: straight-phase HPLC purification followed by $NaBH_4$ reduction afforded the pure hydroxy derivative, which was used as substrate for highly purified L_1 soybean lipoxygenase at pH 9.00. After reduction, a major product (over 90%) was obtained corresponding to 8(*S*),15(*S*)-diHETE (λ_{max} = 259, 269, 280 nm). Appropriate identification was made from identical retention time and coelution with the authentic sample (kindly supplied by Dr. J. Rokach, Merck Frosst, Canada) in reverse-phase HPLC and from

TABLE III

REACTION OF ARACHIDONIC ACID (80 μM) WITH POTATO LIPOXYGENASE. RELATIVE PERCENTAGES OF THE METABOLITES

The relative proportions of the HPETEs are based on the assumption of identical ε (27 500 $M^{-1} \cdot cm^{-1}$) at 237 nm) for each compound. They are calculated from the peak areas of the HPLC chromatogram (either straight- or reverse-phase). The retention times are for reverse-phase HPLC (isocratic elution: 65% CH_3CN in H_2O containing 0.1% CH_3CO_2H). Dihydroxy-arachidonate metabolites were obtained after $NaBH_4$ reduction of the reaction mixture (see text). The relative proportions are based on an identical average ε (40 000 $M^{-1} \cdot cm^{-1}$ at 270 nm). The retention time are for reverse-phase HPLC working in the gradient mode indicated in Materials and Methods. Figures in brackets indicate the relative proportions with respect to all arachidonate metabolites.

	Retention time (min)	Detergent-free homogenate (%)	0.1% Brij 99 homogenate (%)	Purified L_1 5-lipoxygenase
HPETE (90% of reaction mixture)				
5-	15.3	60	65	75 (68)
11-	12.9	20	20	15 (13)
8-	13.6	10	8	5 (4.5)
9-	13.8	10	7	5 (4.5)
Di-HETE (10% of reaction mixture)				
5(S),12(S)-6-*trans*-leukotriene B_4	19.2			22 (2)
12-*epi*,6-*trans*-leukotriene B_4	19.2			22 (2)
5,11- [a]	20.7			22 (2)
5,8- [a]	20.1			22 (2)
	18.7			11 (1)

[a] Tentative assignment (see text).

comparison with the products obtained from the same reactions performed with the racemic alcohol (see Materials and Methods).

Beside HPETEs, L_1 5-lipoxygenase forms simultaneously triene compounds, as is apparent from the ultraviolet spectrum and the HPLC analysis of the crude reaction mixture. The time course of this reaction, followed at 270 nm, is very similar to that obtained at 237 nm, showing in particular a lag phase and a rapid absorbance levelling-off. The initial rate of formation of these products is about 10% that of the HPETEs.

It is noteworthy that the presence of as high as 3 mM $NaBH_3CN$ (able to reduce any free HPETE) does not impede the reaction. This is in favor of immediate conversion of HPETEs at the enzyme active site as soon as they are formed.

That these trienes could be leukotriene B_4 isomers was checked as follows: Incubation of arachidonic acid (5 mg, 80 μM) with 25 units of the purified L_1 was performed for 5–8 min (maximal conversion) at 5°C in 0.2 M acetate buffer (pH 5.5). After reduction and esterification, the mixture of dihydroxymethyl ester derivatives was analyzed on semi-preparative straight-phase HPLC working in a gradient mode, with 280 nm detection. Each peak was collected and the products silylated using standard procedures. GC-MS analysis was done using a specifically designed fused capillary column (see Materials and Methods). The dihydroxy compounds accounted for about 10% of all the metabolites formed, assuming an average molar absorbance coefficient ($\epsilon = 40\,000$ $M^{-1} \cdot cm^{-1}$) [19].

Identification was achieved by monitoring selected ions (GC-MS) and coelution with authentic samples (kindly supplied by Dr. J. Rokach) in HPLC. Using the above-mentioned HPLC system, four peaks corresponding to five compounds were detected at 280 nm in the approximate ratio 1:4:2:2 following the elution order. 5(*S*),12(*S*)diHETE was found to coelute with 6-*trans*-leukotriene B_4 (peak 2, retention time, 19.2 min). These two compounds and the 12-

TABLE IV

INHIBITION OF L_1 5-LIPOXYGENASE-CATALYZED OXIDATION OF LINOLEIC AND ARACHIDONIC ACIDS (IC_{50} VALUES, μM)

IC_{50} values were calculated from the slope of the plot ln V/V_0 versus the concentration of inhibitor, where V and V_0 are the V_{max} measured in, respectively, the presence and absence of inhibitor. Straight lines were obtained from iterative least-squares method. The correlation factors are in parentheses.

Inhibitor	Linoleic acid (150 μM)	Arachidonic acid (100 μM)
ABPH	15 (0.998)	3.9 (0.992)
BPH	34.5 (0.987)	1.4 (0.996)
BW 755C	-	350
NDGA	170 (0.900)	36.5 (0.991)

epi,6-*trans*-leukotriene B_4 (peak 4, retention time, 20.7 min) gave identical ions with only differences in their relative intensity. Prominent fragment ions were found at the indicated *m/e* (followed by proposed identification in parentheses): 293, *M* ($[\overset{+}{C}H_2\text{-}CH = CH\text{-}(CH_2)_4\text{-}CH_3)] - [Me_3SiOH]$); 217, ($Me_3\overset{+}{Si}O = CH\text{-}CH = CH\text{-}OSiMe_3$); 203, ($Me_3\overset{+}{Si}O = CH\text{-}(CH_2)_3\text{-}COOMe$); 129, ($Me_3\overset{+}{Si}O = CH\text{-}CH_2\text{-}CH = CH_2$).

The two other dihydroxylated derivatives were tentatively assigned to 5,11-diHETE (peak 3, retention time, 20.1 min) and 5,8-diHETE (peak 1, retention time, 18.7 min). *

Inhibition of L_1 5-lipoxygenase. Several arylhydrazones have been found to be potent inhibitors of heme [21] and non-heme iron containing enzymes [22,23]. In this laboratory, they have been used to explore the mechanism of L_1 soybean lipoxygenase [24]. Although they are more efficient on the latter enzyme, many of them were found to be potent inhibitors of the potato lipoxygenase. In Table IV are presented IC_{50} values obtained with ABPH and BPH along with those for NDGA and bW 755C as reference inhibitors.

The inhibition depends on the substrate used. We constantly found an order of magnitude decrease in IC_{50} values when using arachidonic instead of lineolic acid. The data show that arylhydrazones are more efficient than classical inhibitors such as NDGA or BW 755C.

Discussion

Based on the use of a non-ionic detergent (0.1% Brij 99), a 5-lipoxygenase has been purified from potato tubers. The procedure yields at least three isoenzymes and the major one (L_1) has been isolated in good yield and with 3–4-fold higher specific activity than that previously reported (250 g of tubers give 20 mg of the enzyme with a specific activity of 140–160 units/mg).

In its reaction with arachidonic acid the present preparation gives 5-, 11-, 8- and 9-HPETEs in relative proportions of 75:15:5:5 together with triene compounds, accounting for about 10% of the whole mixture. The four hydroperoxides have always been found among the reaction products formed from Brij-containing homogenates of eight different tubers varieties. Detergent-free homogenates also give a very similar HPETE distribution (see Table III). The formation of the minor 8- and 9-HPETEs has not been reported before with the potato enzyme. The relative amount of 11- to 5-HPETE (17%) is significantly larger than that found by Shimizu et al. [10] (5%). This raises the question of the presence of a contaminant 11-lipoxygenase in the purified preparation. That 11-HPETE comes from the activity of a 11-lipoxygenase and not from a lack of regioselectivity of the purified 5-lipoxygenase is supported by the following experiment: Potato tubers have been first extracted without any detergent. The material left after gauze filtration and that recovered as pellets after centrifugation of the homogenate were mixed together and submitted to a second homogenization in the same buffer, but containing 0.1% Brij 99. This second extract exhibited a lipoxygenase activity with a significant increase of the 11- to 5-HPETE ratio (35%). Analytical isoelectric focusing supports the presence of a contaminant 11-lipoxygenase whose p*I* is very close to that attributed to L_1 (Fig. 2(B)).

The present preparation exhibits a leukotriene A_4 synthetase activity, as shown by the presence of the two epimers of 6-*trans*-leukotriene B_4 among the metabolites of the arachidonic reaction. This is confirmed by the formation of these sole epimers after anaerobic incubation of 5-HPETE with the purified enzyme. This agrees well with the results of Shimizu et al. [10], who assigned the leukotriene A_4 synthetase activity of their preparation to a 8-lipoxygenase activity demonstrated using bis-homo-γ-linoleic acid, a close arachidonic analogue lacking the C_5-C_6 double bond. Our results show that potato lipoxygenase does form 8(*S*)-HPETE from arachidonic acid. This is in parallel with the results obtained by Borgeat et al. [25] with rabbit polymorphonuclear leukocytes. The presence of both leukotriene A_4 nonenzymatic hydrolysis products and 8(*S*)-HPETE, together with the absence of 12-HPETE, implies that these compounds derive from the abstraction of the same pro-*R*

* Due to the scarcity of the material, complete identification was not feasible. However, both compounds present an ion fragment at *m/e* 203, indicative of 5-hydroxyarachidonate derivatives.

hydrogen atom at C_{10}. After $NaBH_4$ reduction of the reaction mixture, 5(*S*),12(*S*)-diHETE is also a dihydroxylated derivative found in the arachidonic reaction. This compound comes from double oxygenation of the substrate since it is absent from anaerobic incubation of 5-HPETE. The same holds true for the two other minor metabolites detected. The presence of 5(*S*),12(*S*)-diHETE is somewhat unexpected as no 12-HPETE is detected among the reaction products. To check the remaining possibility that 12-HPETE could rapidly be converted to 5,12-diHPETE by the purified L_1, we have looked for a 12-lipoxygenase activity of the preparation. The latter can be ruled out, as no 14,15-leukotriene A_4 hydrolysis products are formed after anaerobic incubation of 15-HPETE with the enzyme [20]. * Taking into account the antarafacial relationship that exists in all lipoxygenase-type reactions [26], this could imply the tilting of the 5-HPETE molecule [27], maybe involving the complementary binding site proposed by Baumann et al. [28].

Based on EPR data, the iron of native L_1 5-lipoxygenase appears in a high-spin ferric state with a pseudo-axial environment. Such a native ferric form has been very recently reported to be partially present (25%) in native lipoxygenases of immature soybean seeds [29]. In our case, the addition of 9-HPOD does not modify the spectrum (the observed symmetrization of the low-field signals is almost certainly due to the isopropanol solvent of 9-HPOD [30]). This rules out the formation of a purple enzyme, as in the case of the L_1 soybean or pea lipoxygenases. Despite its native ferric state, this enzyme presents a lag period for the hydroperoxidation of both linoleic and arachidonic acid. This seems to agree with the proposition that the lag period of lipoxygenases is relevant to hydroperoxide binding to a regulatory site in order to get full catalytic efficiency [18,31].

In summary, we have shown that a 5-lipoxygenase is more readily available from potato tubers when using a non-ionic detergent in the extraction buffer. This suggests that, in potato, this enzyme could be located inside cell membranes. We have found that the pure enzyme is very efficiently stabilized by a high concentration of glycerol. When reacting with its substrates, the L_1 gets inactivated, a behavior common with mammalian lipoxygenases. Although the preparation is not homogeneous, presenting both 5- and 11-lipoxygenase activities (75 and 15%, respectively), it is sufficiently pure to allow mechanistic studies and to provide a good supply of 5-HPETE. Beside its major lipoxygenase activity, this preparation has an intrinsic 8-lipoxygenase activity, as shown by the presence of 8(*S*)-HPETE and both epimers of 6-*trans*-leukotriene B_4 in the arachidonic reaction mixture. At variance with soybean lipoxygenase-1 and pea lipoxygenase, the native form of the potato L_1 5-lipoxygenase is in a high-spin, pseudo-axial ferric state.

* No triene compounds are formed, but 15-HPETE is slowly converted to a 285 nm-absorbing species, which is under investigation.

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  TITLE     Expression, purification, and characterization of a recombinant
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  JOURNAL   Biochem. Biophys. Res. Commun. 243 (2), 438-443 (1998)
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  AUTHORS   Chen,X., Reddanna,P., Reddy,G.R., Kidd,R., Hildenbrandt,G. and
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With Anandamide as Substrate Plant 5-Lipoxygenases Behave like 11-Lipoxygenases

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Anandamide, an endogenous ligand for cannabinoid receptors CB1 and CB2, was incubated with purified 5-lipoxygenases from barley and tomato. This yielded 11*S*-hydroperoxy-5,8,12,14-eicosatetraenoylethanolamide (11*S*-HPANA) as major product (about 70%). This is in contrast with the dioxygenation of arachidonic acid, where 5*S*-HPETE is the major product. This observation implies that the regiospecificity of the dioxygenation, catalyzed by nonmammalian 5-lipoxygenases, is altered by a modification at the carboxylic end of the substrate. Soybean 15-lipoxygenase forms 15*S*-HPANA (95%) and 11*S*-HPANA (5%), and in the second dioxygenation 5,15-diHPANA (45%) and 8,15-diHPANA (55%) are formed. Apparently, the regiospecificity of the soybean 15-lipoxygenase reaction is only slightly affected using anandamide as substrate. © 1998 Academic Press

In the early nineties anandamide (arachidonylethanolamide) was isolated from porcine brain lipids and found to act as a ligand for cannabinoid receptors CB1 and CB2 (1,2). Although structurally distinct from cannabinoids, it shows a similar receptor binding affinity. Anandamide is an arachidonic acid derivative and thereby a substrate for lipoxygenase (3-5) and for cyclooxygenase (6). Lipoxygenases (linoleate: oxygen oxidoreductase 1.13.11.12) are a group of non-heme iron-containing enzymes which catalyze the regio- and stereo-selective dioxygenation of polyunsaturated fatty acids with a (*Z,Z*)-1,4-pentadiene system. Porcine leukocyte 5-lipoxygenase was found inactive towards anandamide (4). In this study the potency of non-mammalian 5-lipoxygenases, like those from barley grains and tomato fruits, to use anandamide as substrate was investigated.

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Abbreviations used: sLOX, soybean lipoxygenase-1; bLOX, barley lipoxygenase-1; tLOX, tomato lipoxygenase; SPE, solid phase extraction; RP-HPLC, reversed-phase high performance liquid chromatography; CP-HPLC, chiral-phase high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; H(P)ANA, *N*-(2-hydroxyethyl)hydro(pero)xyarachidonylamide; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; BHT, 2,6-Di-*tert*-butyl-4-methyl phenol; NDGA, nordihydroguaiaretic acid; CD, circular dichroism.

EXPERIMENTAL PROCEDURES

Materials. Arachidonic acid, NDGA and sodiumborohydride were from Sigma. Anandamide was purchased from ICN biomedicals. Pyridine, 1,1,1,3,3,3-hexamethyldisilazane, chlorotrimethylsilane and BHT were of the highest quality from Aldrich. Palladium on calcium carbonate (5% Pd) was from Acros Organics. Tetrahydrofuran, hexane and methanol were from Biosolve. Barley grains were a gift from Ms. I. Kokkelink (Heineken, Zoeterwoude, The Netherlands).

Enzyme and substrate preparations. Lipoxygenases from barley grains (Triumph), tomato fruits (Trust) and soybeans (white Hilum) were purified as described (7-9). Anandamide and arachidonic acid were purified via solid phase extraction as follows: anandamide or arachidonic acid (5 mg) was dissolved in 2 ml methanol, and 50 ml sodium borate buffer (pH 9.0) was added under continuous stirring. After lowering the pH to 4, the solution was applied to a SPE (Bakerbond, 500 mg, J. T. Baker) column. Autooxidation products were eluted with 5 ml methanol/water (80/20 v/v). Anandamide or arachidonic acid was eluted with 5 ml methanol, concentrated to 2 ml under a stream of nitrogen and BHT was added to a final concentration of 40 μM.

Metabolite generation. Each lipoxygenase was incubated with anandamide or arachidonic acid (final concentration 40 μM; 1 U lipoxygenase per 3 μmol substrate) in 100 mM sodium borate buffer (pH 9.0 for the 15-lipoxygenase and pH 7.0 for the 5-lipoxygenases). The reaction was followed spectrophotometrically at 236 nm. After completion, the pH was lowered to 4 and the products were purified with SPE (Bakerbond, 500 mg, J. T. Baker) and then analyzed by HPLC and/or GC/MS.

Spectrophotometric assay. Spectrophotometric assays were performed by continuously monitoring the change in absorbance at 236 nm on a Hewlett-Packard 8452A diode array spectrophotometer. A molar absorbance of 23000 M^{-1} cm^{-1} at 236 nm was used for H(P)ETEs and H(P)ANAs (3) and 25000 M^{-1} cm^{-1} at 234 nm for HPODs. For 8,15- and 5,15-diHPANA molar absorbances of 40000

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M^{-1} cm^{-1} at 269 nm and 33500 M^{-1} cm^{-1} at 243 nm were used respectively (10).

HPLC. Reaction products of anandamide and arachidonic acid with the various lipoxygenases were analyzed on a Hewlett-Packard 1090 LC HPLC system equipped with a HP 1040A diode array detector and an HP7994A analytical workstation. RP-HPLC was carried out on a Cosmosil 5C18 AR column (5 μm, 250 × 4.6 mm, Nacalai Tesque) with tetrahydrofuran/methanol/water/ acetic acid (25/40/35/0.1 v/v/v/v) as solvent at a flow rate of 1 ml/min. Chiral separations of the sodiumborohydride-reduced anandamide metabolites were carried out on a Chiralcel OD-R column (5 μm, 250 × 4.6 mm, Daicel) with methanol/water/acetic acid (75/25/0.1 v/v/v) as eluent at a flow rate of 0.5 ml/min. In separate experiments anandamide metabolites generated by the different lipoxygenases were isolated by RP-HPLC and reduced with an excess of sodiumborohydride at 0°C for 30 min. After lowering the pH to 4, the resulting hydroxy-anandamide metabolites were purified by SPE (Sep-Pak tC18 Vac, 50 mg, Waters), and analyzed by CP-HPLC.

Circular dichroism spectroscopy. CD-spectra of the RP-HPLC purified HANAs (about 40 μM HANA in methanol, optical pathway 1.0 cm) were recorded on a JASCO J-600 spectropolarimeter from 210 to 270 nm. Typically 10 spectra (resolution of 1 nm, scan speed 10 nm/min) were accumulated and corrected for an independently recorded baseline of 40 μM anandamide in methanol.

Gas chromatography-mass spectrometry. Anandamide metabolites prepared with bLOX and sLOX were reduced with an excess of sodiumborohydride at 0°C for 30 min and after lowering the pH to 4 purified by SPE (Sep-Pak tC18 Vac, 50 mg, Waters). The hydroxy-

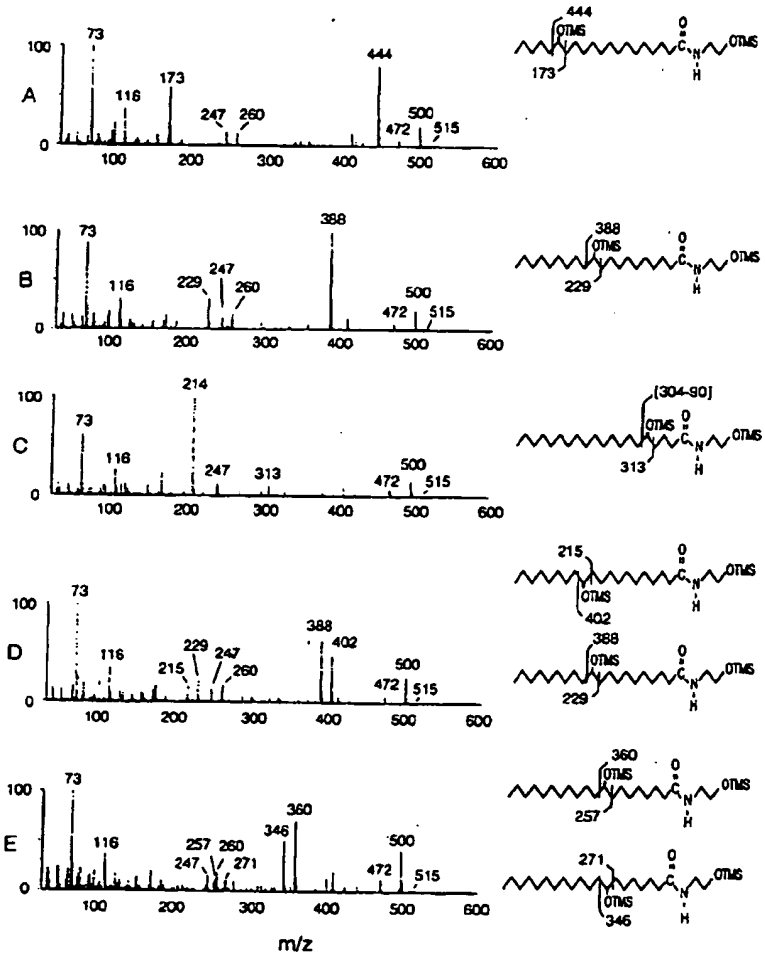

FIG. 1. Mass spectra and deduced structures of the monoHPANAs generated by the different lipoxygenases. Structures of 15-HPANA (A), 11-HPANA (B), 5-HPANA (C), 12-HPANA contaminated with 11-HPANA (D), and 8- and 9-HPANA (E) were deduced from the spectra of the reduced, hydrogenated and trimethylsilylated HPANA derivatives. Insets represent the deduced structures and their main fragmentation patterns.

TABLE 1

Relative Reaction Rates of sLOX, bLOX, and tLOX with Arachidonic Acid and Anandamide Compared with Linoleic Acid

Substrate	Enzyme		
	sLOX	bLOX	tLOX
Linoleic acid	100[a]	100	100
Arachidonic acid	160	15	12
Anandamide	88	33	15

[a] Reaction rates were measured spectrophotometrically by continuously monitoring the increase in absorbance at 234 nm for linoleic acid and at 236 nm for arachidonic acid and anandamide, with 40 μM substrate in 100 mM borate buffer (pH 9.0 for sLOX and pH 7.0 for bLOX and tLOX). The values indicate the reaction rates (linear parts of the progress curves) relative to linoleic acid.

anandamide metabolites were eluted with methanol, dried under a nitrogen stream, redissolved in 1 ml hexane and hydrogenated with a catalytic amount of palladium on calcium carbonate (5% Pd) in a hydrogen atmosphere. After 30 min, the catalyst was removed by filtration over a pre-washed (hexane) piece of cotton wool. Hexane was evaporated under a nitrogen stream and silylation reagent (50 μl; pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane (5/1/1 v/v/v)) was added. After 30 min at room temperature, the silylation reagent was evaporated under a stream of nitrogen and the residue was redissolved in 20 μl hexane. An aliquot was analyzed by GC/MS (Carlo Erba GC 8060 with a Fisons MD 800 mass detector) equipped with a CP-Sil 5 CB-MS column (25 m × 0.25 mm × 0.25 μm, Chrompack). The column temperature was held at 200°C for 1 min, increased in 13 min to 330°C and held at this temperature for 2 min. Mass spectra were recorded under electron impact with an ionization energy of 70 eV.

RESULTS

Anandamide was incubated with three plant lipoxygenases and the reactions were followed spectrophotometrically. In comparison to arachidonic acid the reaction rates of sLOX, bLOX and tLOX with anandamide were halved doubled or equal, respectively (Table 1). All reactions were inhibited in the presence of NDGA, a known LOX inhibitor (11).

The reaction products were reduced, hydrogenated and trimethylsilylated, without further purification. With GC/MS the monoHPANA derivatives are separated into three peaks which can selectively be monitored at *m/z* 500, $[M\text{-}CH_3]^+$. The first peak contains 5-HPANA, the second peak 12-, 11-, 9- and 8-HPANA, and the third peak 15-HPANA (data not shown). The mass spectra of the anandamide metabolites are given in Fig. 1 and the most abundant fragmentations are listed in Table 2. The reduced, hydrogenated and trimethylsilylated monoHPANA derivatives typically show four main ion peaks, namely, *m/z* 73, the TMS fragment, *m/z* 500, the $[M\text{-}CH_3]^+$ fragment, and two ion peaks around the dioxygenated C-atom, where the fragmentation towards the *N*-2-ethyl-OTMS part is the most abundant. The only exception is 5-HPANA, where the fragmentation towards the *N*-2-ethyl-OTMS part yields *m/z* 214 instead of *m/z* 304, due to the loss of HOTMS. The hydrogenation step in the derivatisation scheme has the advantage that the structures show characteristic ion peaks that via selective ion monitoring the different anandamide metabolites can easily be recognized.

Surprisingly, the major product of the anandamide dioxygenation by plant 5-lipoxygenases is 11-HPANA, whereas with arachidonic acid as substrate 5-HPETE is formed. Reaction products were quantified by RP-HPLC (Fig. 2, Table 3) and identified by GC/MS.

The assignment of the absolute configuration was based on CD-spectroscopy in combination with chiral separations. The CD-spectra of 15-HPANA (sLOX), 11-HPANA (bLOX), and 5-HPANA (bLOX) showed a positive Cotton effect, so they have predominantly *S*-configuration (12). On this basis chiral separations were used to obtain the accurate *R/S* ratios of the anandamide metabolites (Table 3). The regiospecificity of the reaction of bLOX with anandamide was not pH dependent, as incubations between pH 5.2 and 9 gave identical RP-HPLC patterns.

Soybean lipoxygenase, at relatively high enzyme concentrations, is capable of converting arachidonic acid into 8,15- and 5,15-diHPETE (10). For anandamide, comparable results have been reported based on absorbance measurements (5). Here, the two doubly dioxygenated anandamide metabolites were separated by RP-HPLC, analyzed by GC/MS and the mass spectra are shown in Fig. 3. These compounds were identified on the basis of the $[M\text{-}CH_3]^+$ fragment (*m/z* 588), and show also the characteristic ion peaks around the dioxygenated C-atoms (Table 4). The compounds were identified as 8,15- and 5,15-diHPANA and are formed in a molar ratio of 55:45 as determined by RP-HPLC (data not shown).

DISCUSSION

Fatty acid ethanolamides, containing one or more 1*Z*,4*Z*-pentadiene systems, have been described as

TABLE 2

Characteristic Mass Fragments of the Reduced, Hydrogenated, and Trimethylsilylated Anandamide Derivatives (*cf.* Fig. 1)

Anandamide metabolite	Characteristic fragment ions (*m/z*)		
	$[M\text{-}CH_3]^+$	Dioxygenated C-atom	TMS
15-HPANA	500	444; 173	73
12-HPANA	500	402; 215	73
11-HPANA	500	388; 229	73
9-HPANA	500	360; 257	73
8-HPANA	500	346; 271	73
5-HPANA	500	214; 313	73

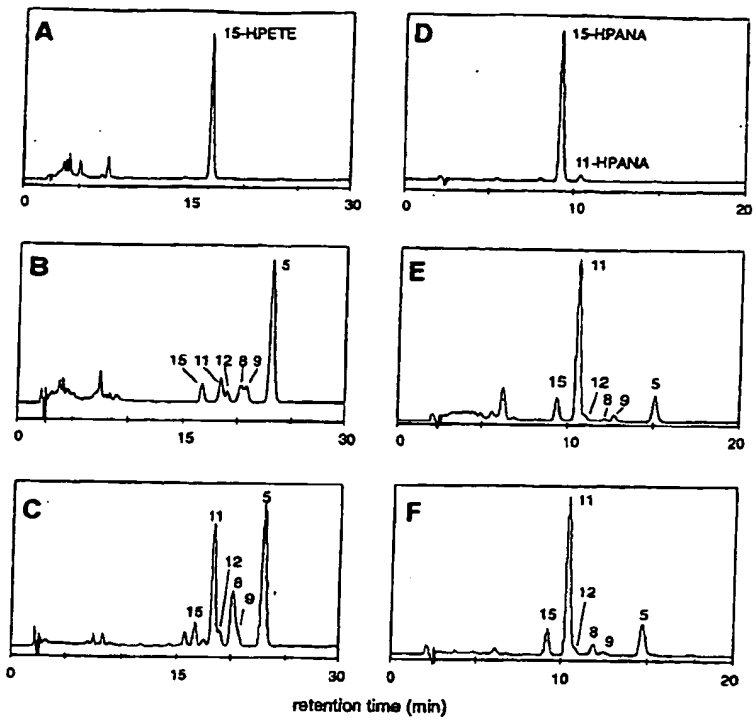

FIG. 2. Reversed-phase HPLC analysis of reaction products formed from arachidonic acid and anandamide with various lipoxygenases monitored at 236 nm. Arachidonic acid at 40 μM was incubated with sLOX (A), bLOX (B) and tLOX (C). Anandamide at 40 μM was incubated with sLOX (D), bLOX (E), and tLOX (F). The elution positions of 8- and 9-HPANA may be interchanged.

suitable substrates for lipoxygenases. Native 15-lipoxygenases from soybeans, rabbit reticulocyte, and porcine leukocyte 12-lipoxygenase show the same regiospecificity towards anandamide as with arachidonic acid (3,4). However, porcine leukocyte 5-lipoxygenase has been reported to show no activity towards anandamide

TABLE 3

The Product Specificity (Percentage) for Arachidonic Acid and Anandamide by Soybean Lipoxygenase-1 (sLOX), Barley Lipoxygenase-1 (bLOX), and Tomato Lipoxygenase (tLOX) with Enantiomeric Composition of the Major Anandamide Metabolites

Position OOH	sLOX		bLOX		tLOX	
	HPETE	HPANA	HPETE	HPANA	HPETE	HPANA
15-	100[a]	95 (95/5)[b]	6	9 (60/40)	5	10 (50/50)
12-	—	—	4	1	3	2
11-	—	5 (80/20)	10	72 (95/5)	30	65 (95/5)
9-	—	—	7	3[c]	3	2[c]
8-	—	—	7	1[c]	19	5[c]
5-	—	—	66	14 (95/5)	40	16
	(80/20)					

[a] Isomeric ratio of the dioxygenation products by the three lipoxygenases, as determined by RP-HPLC.
[b] The enantiomeric ratios (*S/R*) of the main products, as determined by chiral separations in combination with CD spectroscopy.
[c] The distributions of 8- and 9-HPANA may be interchanged.

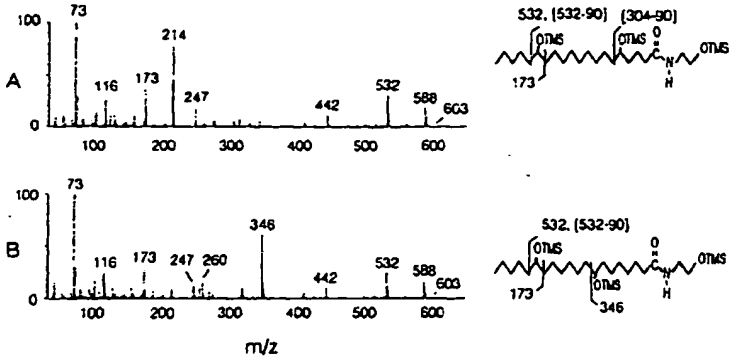

FIG. 3. Double dioxygenation of ANA by sLOX. Mass spectra of the reduced, hydrogenated and trimethylsilylated 5,15- (A) and 8,15-diHANA (B) derivatives. Insets represent the deduced structures and their main fragmentation patterns.

(4). Interestingly, we observed here that 5-lipoxygenases from barley grains and tomato fruits converted anandamide into 11*S*-hydroperoxy-5,8,12,14-eicosatetraenoylethanolamide (11S-HPANA). With arachidonic acid as substrate these 5-lipoxygenases yield 5*S*-HPETE as the major product, while they are inactive towards methyl arachidonate. This implies that with anandamide as substrate for plant 5-lipoxygenases hydrogen abstraction occurs at C-13, whereas with arachidonic acid it occurs at C-7.

In a study with four different *N*-linoleoyl amides, the dioxygenation by soybean 15-lipoxygenase showed similar regio- and stereoselectivities, compared to linoleic acid (13). The results reported here show that also the double dioxygenation of anandamide by soybean 15-lipoxygenase is similar to that of arachidonic acid.

Recently, the X-ray crystallographic data of a mammalian 15-lipoxygenase have been published together with a hypothesis to explain the positional specificity of mammalian lipoxygenases (14,15). In this model the substrate is oriented with its methyl end towards the active site for all different lipoxygenases. Our data show that anandamide enters the active site of the plant 5-lipoxygenases, but the observed regioselectivity suggest that the presence of the ethanolamide group prevents the substrate from penetrating deeper into the active site cavity, to allow the C-5 regioselectivity.

The observations reported here demonstrate that the regiospecificity of the 5-lipoxygenase reaction changes through modification of the carboxylic terminus of the substrate, while it has been shown earlier to change after site-directed mutagenesis (16).

TABLE 4

Characteristic Mass Fragments of the Reduced, Hydrogenated, and Trimethylsilylated Doubly Dioxygenated Anandamide Metabolites (cf. Fig. 3)

	Characteristic fragment ions (*m/z*)		
Anandamide metabolite	$[M\text{-}CH_3]^+$	Dioxygenated C-atoms	TMS
8,15-diHPANA	588	532; 442; 346; 173	73
5,15-diHPANA	588	532; 442; 214; 173	73

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